(54) Title: AGGREGATION-BASED ASSAYS

(57) Abstract: A method for measuring aggregation by the detection of individual particles (30) is disclosed. The method is adaptable for screening compounds, detection of low levels of a target (e.g. small molecules, large molecules, cells, or microbes), competitive binding assays and other assays. If the assay mixture contains a targeted compound (18), the particulate substrate (20) will aggregate to form a macroparticle (30). One of the components of the assay mixture is labeled with an optically detectable label (14). The macroparticle (30) has an associated macroparticle optical signal which would be identified by optical analysis of the assay mixture.

Description

AGGREGATION-BASED ASSAYS

5   TECHNICAL FIELD

This invention relates to an assay to detect a
target of interest through the formation of particles
within a detectable size range.

10   BACKGROUND OF THE INVENTION

Detection of small amounts of compounds is
required for current analytical and diagnostic assays.
For example, small amounts of substances present in pa-
tient samples, such as blood or tissue, serve as an indi-

15   cation of disease states.. Monitoring of substances in
patient bodily fluid, such as pharmaceuticals, narcotics,
toxins, hormones, antibodies, etc. is required for diag-
nosis and treatment of patient conditions. Numerous
samples containing minute quantities of substances must

20   be tested. The sample analysis must be rapid, sensitive,
and specific for the substances tested.

Immunoassays are often used to detect small
amounts of a target substance. Examples include radio
immunoassay and enzyme immunoassay both of which have

25   been used as sensitive assays for specific antigens.
These assays allow detection of hormones, pharmaceu-
ticals, and other small compounds present in bodily flu-
ids at very low levels (e.g. picomoles/liter).

Immunoassays may either be heterogeneous (i.e.

30   a separating phase is used to remove the bound detectable
target from the assay mixture) or homogenous (i.e. the
sample is directly detected in a native sample without a
separation step). Because heterogeneous assay does not
allow detection in a native sample, these assays present

35   certain drawbacks. The washing or separating steps re-

quire additional material and experimentalist time and
may alter the cell signal. In contrast, homogenous as-
says save time and resources by not requiring a separa-
tion step. Additionally, detection in a native sample
5   reduces risk of error or contamination resulting from
each manipulation step.

Aggregation assays provide one method for ho-
mogenous detection of compounds with the high sensitivity
required to small quantities of compounds in solution.
10   In aggregation assays, a solution of binding agents is
mixed with a test sample. If the targeted compound is
present, the compound will interact with the binding
agent and produce a characteristic aggregation pattern.
Such an aggregation pattern observed on a macro scale
15   could be observed as a "clumping" of the aggregating
particles. Two commonly practiced types of aggregation
assays are latex-based tests and blood cell-based tests.

Latex agglutination

20   Latex agglutination assays generally detect the
presence of an antigen by using a competitive binding
assay system. An antigen and a latex bead having an
associated antigen-analog or conjugate of the antigen
compete to bind to the binding sites of the antigen spe-
25   cific antibody. If no antigen is present in the test
mixture, the antibody binds to the antigen analog or
conjugate of the antigen analog to form clumps that may
be visually detected. If the antigen is present the
antigen will compete with the attached compounds on the
30   latex beads for the antibody binding sites. This pre-
vents the reaction of the latex beads with the antibody,
preventing aggregation of the latex beads. In this as-
say, the reaction mixture is generally visually observed
to determine assay agglutination. Agglutination indi-
35   cates the presence of the antigen of interest.

Various devices have been developed for the
elimination of the subjective determination of the re-
sults of the latex-based assays. For example, U.S. Pat.
No. 5,491,095 to Bepko et al. discloses an automatic
5   reader for latex-based agglutination reactions. The
reader directs light onto a flowing reaction mixture. A
plurality of detectors positioned at a number of loca-
tions detect light that has passed through the sample.
The variation in the amplitude detected by each detector
10   indicates whether the sample is aggregated or uniform.
The amplitude variation must exceed a threshold measure-
ment to be detected.

An alternative assay described in U.S. Pat. No.
5,489,537 uses various dyes in a process that automates
15   the detection of latex aggregation. In this assay, a
test sample is incubated with a suspension of latex beads
and a detectable colloidal dye. The latex particles are
conjugated to a binding agent for binding a target anti-
gen. If the targeted antigen is present, the latex beads
20   aggregate and some of the aggregated latex particles
entrap the colloidal dye. This reaction mixture is then
brought into contact with a porous membrane. The pores
of the membrane allow the non-aggregated latex particles
to flow through but trap the aggregated latex particles
25   containing the colloidal dye. The amount of colloidal
dye remaining in the membrane is measured. The strength
of this measurement is an indication of the amount of
aggregation which in turn indicates the amount of the
substance of interest.
30

Blood Based Aggregation
Hemagglutination assays use erythrocytes (such
as sheep red blood cells) and anti-erythrocyte antibodies
in an indicator system. The red cells, coated with the
35   antibody are reacted with the sample. Antigens with

binding agents for multiple epitopes cause red cells to aggregate to form agglutinated masses. It is also possible to design an indirect assay in which the sample compound competes with the red cells for the binding to

5   binding agents. In the absence of competitive binding, agglutination is observed.

U.S. Pat. No. 5,768,407 to Shen et al. describes a method for automating the detection of aggregation in a blood-based aggregation assay. In this method

10   an illuminated image is produced and detected as an array of pixels, with each pixel having a value representative of the intensity of the illuminated image on the pixel. The values are subsequently processed, based upon a stored set of variables, to determine if aggregation has

15   occurred and to classify the aggregation if it has occurred.

U.S. Pat. No. 5,583,003 to Hillyard et al. describes a direct agglutination measurement using blood cells. In this reference two reagents are provided, each

20   of which binds to a non-overlapping epitope of a targeted antigen. In two separate assay mixtures, each of the reagents is separately mixed with a test compound that may contain the antigen. The two assay mixtures are then combined and the resulting agglutination indicates the

25   presence of the antigen in the sample.

Although the present assays are capable of detecting low levels of a target antigen in a test sample, in general relatively large amounts of reagents are required to test for a target antigen. In addition, some

30   of the present methods remain subjective and do not provide an automatic indication of the amount of the antigen of interest present in the test sample.

The present aggregation assays are generally designed to detect small compounds, such as narcotics or

35   hormones. It would be advantageous to adapt present

aggregation assays to use currently available optical detectors to detect aggregation on a very small scale. Further utility would be added if the detection system is sufficiently flexible to be used for aggregation assays

5   that could detect small compounds, large compounds and bacterial or other microbial cells.

It is an initial object of the invention to provide an agglutination assay for the detection of antigens and the screening of compounds that is able to de-

10   tect small amounts of compounds in a homogenous assay. The assay should be adaptable both for diagnostic tests as well as for the screening of a library of compounds to determine the interaction of the compounds with a binding agent. It is a further object that the test be performed

15   in microvolume reactions. It is a further object that the present assay be adaptable for use on presently available optical detection systems. It is a final object of the invention that the assay be able to detect both small and large compounds as well as bacterial cells

20   in an aggregation assay.

SUMMARY OF THE INVENTION

The above objects are achieved through an aggregation assay which uses an optical system that can

25   optically distinguish labeled macroparticles, preferably in a homogenous assay. This assay requires an optical system that may discriminate the macroparticles by detection of an associated optically detectable label.

In one embodiment of the assay, at least one

30   microparticle and one binding agent are provided in an assay mixture. The binding agent may be present on the microparticle or could be a reagent free in solution. Either the microparticle or the binding agent or the antigen of interest is labeled with an optically detect-

35   able label. If the antigen of interest is present, the

antigen will compete for the binding sites on the binding
agent. The assay mixture is subsequently optically in-
terrogated. In one embodiment, this is effected by plac-
ing the assay mixture into a static chamber which is

5   scanned in a limited depth of field. The optical scanner
detects the optically detectable label and/or has a par-
ticle size gating function that detects the size of
·particles. The degree of formation of macroparticles or
signal intensity in the presence of the test sample com-

10   pared to a control with no antigen is an indication of
the amount of antigen present in the sample.

        In one embodiment, a microparticle having an
associated binding agent, and an antigen labeled with an
optically detectable agent are combined in an assay mix-

15   ture with a test sample. The microparticle binding agent
binds to an epitope of the target antigen (usually a
large molecule) conjugate. Additional microparticles
bind to other epitopes on the antigen. This linking of
microparticles produces agglutination and formation of

20   macroparticles. Macroparticles may then be detected by
an optical system that detects the enhanced signal from
the localized concentration of optically detectable label
present on macroparticles. If the target antigen is
present in the test sample, macroparticles will be formed

25   similarly; however signal will vary depending on the
concentration of the target antigen in the test sample.
Additionally, the optical detection system may distin-
guish particles of the macroparticle size from
microparticle.

30   Small antigens can be detected in a similar
manner. Generally, small antigens are coated on optic-
ally detectable particles. A binding agent that binds
the antigen is included in the assay mixture, either in
solution or coated onto a particle in suspension.

35   Agglutination will occur if a sufficient amount of free

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antigen is present in the assay mixture. Several varia-
tions to this protocol are possible. Optical detection
is important; at least one reagent of the assay should be
optically detectable.

5   In one embodiment, multiple assays may be per-
formed in a single assay mixture and analyzed in a single
scan. The optical signal from each assay would provide a
signal which the optical detection system could use to
distinguish the assay results. The unique signal could
10   be the emission wavelength of an associated dye,
macroparticle size or shape or other feature detectable
by the optical system. The ability to perform multiple
assay in a single assay mixture could also be adapted for
analysis of interaction of multiple epitopes of a large
15   molecule to be analyzed in a single assay.

BRIEF DESCRIPTION OF THE DRAWINGS
          Fig. 1A is a representational illustration of
the combination of a microparticle bead conjugated to
20   binding agents and a detectable label. This combination
causes aggregation resulting in the formation of a macro-
particle when free antigen is added to the assay mixture.
          Fig. 1B is a representational illustration of
the combination of a microparticle bead having an asso-
25   ciated antigen with a binding agent having two binding
sites. The binding agent has an associated detectable
label. This combination causes aggregation.
          Fig. 1C is a representational illustration of
the combination of Fig. 1B with the assay mixture that
30   further includes analyte free in solution. In this com-
bination, the analyte competes for the binding sites on
the binding agent, inhibiting aggregation.
          Fig. 1D is a representational illustration of
rosetting.

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Fig. 1E is a representational illustration of
macroparticle formation in an assay using antibodies as a
binding agent.

Fig. 1F is the illustration of Fig. 1E in which
5   free antigen reduces the macroparticle signal.

Fig. 2A is a representational illustration of
bacterial cell aggregation.

Fig. 2B is the representation of the assay of
Fig. 2A in which aggregates did not form.

10   Fig. 3 illustrates a schematic of an optical
system that could be used in conjunction with the present
invention.

Fig. 4 illustrates a representation of a scan
of an assay mixture containing microparticles and macro-
15   particles and the corresponding detection signals.

Figs. 5A-5F are dot plots showing the size and
intensity of detection events in a streptavidin-biotin
binding system measured on the IMAGN 2000™.

Fig. 6 is a dot plot of the flow cytometry
20   measuring the fluorescence intensity and light scattering
in an assay mixture containing no Streptavidin Blue bead.

Figs. 7A-7D are dot plots of the flow cytometry
analysis of the assay mixtures described in relation to
Table 3.

25   Figs. 8A-8D are dot plots of the IMAGN 2000
analysis of the assay mixtures described in relation to
Table 3.

Figs. 9A-9E are dot plots of the IMAGN 2000
analysis of the assay mixtures described in relation to
30   Table 5.

Figs. 10A-10F are dot plots of the IMAGN 2000
analysis of the assay mixtures described in relation to
Table 7. In the present assay the formation of indivi-
dual macroparticles is detected. Each macroparticle is

composed of two or more smaller particles linked by a
binding agent.

BEST MODE FOR CARRYING OUT THE INVENTION

5   Detection is effected by an optical detection
system that preferably detects both the size of the
macroparticle and the strength of a detectable label
associated with the macroparticles.
           For the present invention, a microparticle is
10   defined as a single particle, cell, or compound.
           A macroparticle is defined as an aggregation of
a plurality of microparticles. A macroparticle produces
an optical signal intensity greater than the signal pro-
duced by a microparticle. The size of the macroparticle
15   will generally be between 0.01 µm and 50 µm.
           The macroparticles are sufficiently dilute and
sufficiently small that an optical detection system may
detect the associated signal from a macroparticle as
single, discrete detection events. In addition, the
20   macroparticle must provide a sufficiently strong optical
signal to distinguish the macroparticle from both
microparticles and any optical background.
           In one embodiment the macroparticle is detected
by optical scanning systems which scan an assay mixture
25   in a limited depth of field. The width of the
macroparticle is preferably between 0.5 and 10 times the
width of the beam spot. It is also preferred that the
optical scanning system recognize a difference in signal
profile between a microparticle optical signal event (if
30   detectable by the system) and the macroparticle optical
signal event.
           In addition, the signal intensity from the
detected macroparticle may be used to detect whether a
labeled compound or competitive binding compound is pres-
35   ent in the assay mixture. In addition multiple assays

may be performed in a single assay mixture. Each assay
could include a different optically detectable label
associated with each assay macroparticle. The optical
detection system could be used to distinguish two or more

5   optically detectable labels, each associated with a spe-
cific assay. Alternatively the macroparticle could be
distinguished by size or other means.

In the present invention, the binding of a
binding agent to a binding site produces the macroparti-

10   cle. Binding sites may include a surface bound antigen,
a surface bound receptor, a surface bound antibody, or
any other class of compound which may serve as a site for
a binding event. In addition, an enzyme and associated
substrate could serve as the binding agent/binding site

15   pair. Binding sites may be on the surface of a bead or
cell collectively comprising a microparticle. Alterna-
tively, receptor or antibody having multiple binding
domains could act as binding sites.

Binding agents are any compound or complex

20   which binds to a binding site to enable aggregation. The
binding agent may be used as a reagent free in solution,
or attached to the surface of a cell or bead. A binding
agent may be multivalent, i.e. able to bind at least two
sites, allowing linkages. Examples of binding agents

25   include antibodies, enzymes, receptors, receptor domains,
etc.

I. Assays

The assay of the present invention is illus-

30   trated in the representational diagrams of Fig. 1.   In
Fig. 1A a representational diagram illustrates a first
method of detection of the presence of an antigen through
the use of a bead and an associated binding agent. Bead
12 with a binding agent 16 covalently bound to the bead

35   surface is provided. The binding agent is specific for

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antigen 18. Also associated with the bead is a detect-
able labeling agent 14. This coated bead comprises
microparticle 20. When the microparticle 20 is combined
with antigen 18 in an assay mixture, the antigen 18 binds

5   to the binding sites. The antigen has multiple copies of
one epitope that binds to the binding sites. The antigen
may be bound to multiple binding sites, with each binding
site binding one of the antigen's epitopes. Multiple
microparticles aggregate as the binding agents on each

10   microparticle bind to one of the repeated epitopes on the
antigen, linking the microparticles together. The aggre-
gation produces a macroparticle 30.

       The macroparticle may be detected by scanning
the assay mixture to detect a localized concentration of

15   signal from the detectable label associated with the
presence of a macroparticle 30. In addition, the optical
system could sort the particles by size to distinguish
particles of the size of a macroparticle from the smaller
sized microparticles. In this assay a macroparticle will

20   form only in the presence of antigen. Thus the detection
of macroparticles, the intensity of the signal from the
associated optical labels detected from macroparticles,
and the size (indicated by signal profile) of macroparti-
cles are all indications of the presence of the antigen

25   in the assay mixture. These combined measured signals
(collectively the macroparticle signal) may increase as
the amount of antigen in the assay mixture increases,
depending on assay design. The macroparticle signal
measured from assay mixtures containing various known

30   concentrations of the antigen may be compared to the
signal measured from an assay mixture containing an un-
known concentration of the antigen. This comparison
determines the unknown antigen concentration.

       In this embodiment of the assay, the antigen

35   must have two binding sites for aggregation to occur.

This assay is suitable for detection of large molecules
and cells.   Two binding reactions are required for the
aggregation of microparticles, giving the assay high
specificity.   A single, non-specific binding event would

5   be insufficient to create aggregated macroparticles.   In
this respect, this embodiment of the assay is similar to
the sandwich type immunoassays.

Fig. 1B is a representational illustration of
an alternative assay.   In this assay, a bead 12 is coated

10   (e.g. by covalent bonding) with an antigen 18.   The bead
and associated antigen comprise a microparticle 20.
Microparticle 20 is combined in an assay mixture with a
soluble binding agent 16 conjugated to a detectable label
14.   The binding agent 16 binds to antigen 18 attached to

15   the surface of the bead.   The binding agents 16 have
multiple attachment sites, allowing the binding agent to
bind to the antigen associated with multiple micropar-
ticles.   This results in the formation of macroparticle
30 comprised of multiple antigen coated beads linked by

20   the binding agents.   The presence of a macroparticle will
correspond to a localized concentration of the detectable
label associated with the binding agent.   The macroparti-
cle may be detected by detection of a localized concen-
tration of the detectable label.   If the free antigen is

25   present in the sample, then it will bind to binding agent
and fewer macroparticle aggregates will be formed.

The assay of Fig. 1B may be used for the detec-
tion of an antigen in an assay mixture.   This is illus-
trated in Fig. 1C.   In this assay, microparticle 20 com-

30   prised of a bead 12 with an associated surface bound
antigen 18 is combined with the binding agent 16 having
an associated detectable label 14.   In addition, the
assay mixture also contains antigen 18 free in solution.
In this assay, some of the antigen that is free in solu-

35   tion will bind to binding agent 16.   The binding agents

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that are bound to the antigen that is free in solution
will not be available to bind to particle associated
antigen. When less binding agent is available to bind
particles, aggregation of the particles will be inhib-
5   ited. When aggregation is inhibited, there will be re-
duced detection of localized concentration of the detect-
able binding agents. The detectable label associated
with the binding agent that is free in solution will be
detected as part of the optical background. In the assay
10   embodiment depicted in Figs. 1B and 1C, when the target
antigen is not present as a soluble compound, the forma-
tion of macroparticles, as indicated by the macroparticle
signal, will be greatest. The macroparticle signal will
decrease as additional soluble antigen is added to the
15   assay mixture. In this way, the amount of an antigen in
a test sample may be determined by detecting the inhibi-
tion of the macroparticle formation and attendant
macroparticle signal.

In the assay depicted in Fig. 1A, aggregation
20   occurs when the antigen was bound by two binding agents.
In contrast, in the assay depicted in Figs. 1B and 1C
aggregation occurs when the antigen associated to each
bead is bound by the binding agent at a single binding
site.

25   In another embodiment, the presence of a bind-
ing agent may be detected through "rosetting". In Fig.
1D, fluorescent beads 32 have a surface bound antigen 34.
When an excess concentration of the beads are combined
with a binding agent 36, the binding sites 38 bind the
30   surface antigen 34. The resulting "rosette"
macroparticle 39 may again be detected by size gating and
detection of the increased optical signal. In this assay
the limited number of binding sites on the binding agent
limits the total size of the final aggregated macroparti-
35   cle. In example 1D a tetrameric binding agent 36 has

four binding sites 38 and may bind four beads 32. This
assay may be adapted for screening liquid samples to
determine if a soluble target compound is present in the
sample. If the antigen is present in solution, it would
5   bind to the binding agent. Fewer beads would bind to the
binding agent, inhibiting the rosette formation. Subse-
quent analysis of the assay mixture would detect fewer
rosettes with reduced signal intensity.

        The assay could also be modified for use as a
10   competitive binding assay. In such an assay, a screened
compound from a compound library would be combined with
the assay mixture. If the compound has an affinity for
the binding sites, the macroparticle or rosette formation
would be inhibited if the screened compound binds to the
15   binding agent. If the screened compound did not have an
affinity for the binding agent, no inhibition of rosette
formation would occur.

        The present invention may use an antibody as a
binding agent. Antibodies are commercially available,
20   may have highly specific binding, high binding constants
and may be conjugated to dyes and surface associated
functional groups.

        In Fig. 1E, a fluorescent bead 40 has a surface
associated antigen 42. When bead 40 is combined with
25   antibody 44, antibody binding domain 46 may bind to anti-
gen 42. If antibody 44 has binding regions that may bind
to two separate antigens, one antibody may serve as a
link between beads, causing formation of macroparticles.
Macroparticle 48 may then be detected, again by size
30   gating and optical label intensity detection. Fig. 1F
depicts the same assay with free analyte 49 present in
the assay mixture. In this assay macroparticle 48 signal
intensity and size would be diminished because fewer
beads would be incorporated into the macroparticles.

Instead, the antibodies would bind to the soluble analyte.

The assays of Figs. 1A-1F are useful in screening samples to determine the amount of antigen present in

5   the sample. The antigen to be detected may be a narcotic, a pharmaceutical agent, a hormone, a protein or glycoprotein or cells (e.g. microbes).

In addition to detection of specific compounds, this assay may be used to screen a compound library to

10   determine if the component compounds in the library bind to the binding agent used in the assay. This screening may be used to determine if the compounds have an affinity for the binding agents. If the amount of the compound from the library is known, detection of

15   macroparticle formation and associated optical signal may serve as an indication of the strength of the affinity of the screened compound for the binding agent. Any of the assays illustrated may be used either to determine the amount of an antigen in an assay mixture or to screen a

20   library of compounds to determine binding activity. U.S. Pat. No. 5,876,946, hereby expressly incorporated by reference herein describes techniques useful for screening chemical libraries.

In the assays of Figs. 1A-1F, a number of dif-

25   ferent beads or other microparticle supports may be used. Latex beads between the size of 0.01 µm and 10 µm are suitable. The preferred size is between 0.05 to 2 µm, and most preferably is 0.2 µm in size. Both functionalized beads (beads with functional groups such

30   as amino, hydroxyl, carboxyl, or halo groups bound to the surface of the bead) and non-functionalized beads are adaptable to the present assay as long as appropriate methods for conjugating fluorescent dye, analyte and/or binding agents to beads are developed. Fluorescent beads

35   are currently available from various commercial sources

such as Spherotech (Libertyville, Ill.), Bangs Laborato-
ries (Fishers, IN); Molecular Probes (Eugene, OR). Suit-
able beads include latex polystyrene beads, either un-
coated or coated with avidin having 10^6 binding sites per
bead. In addition to latex beads, suitable beads may be
made of cellulose, silica, glass, grafted co-polymers,
polyacrylamide, dimethyacrylamide, or other suitable
materials. Beads of various sizes labeled with a fluo-
rescent or other optically detectable dye are available
from Molecular Probes (Eugene, Oregon) and Spherotech
(Libertyville, IL). These beads are available either
with or without associated functional groups. Addition-
ally these beads are available coated with biotin,
streptavidin, NeutraLite avidin and Protein A.

In one embodiment of the assay the bead was
coated with a receptor or antibody that functions as the
binding agent. Alternatively, the receptor or antibody
may be free in the assay mixture and function as binding
agents that attach to binding conjugates (binding sites)
present on the surface of a bead. Numerous binding
agents are adaptable to these assays. Such binding
agents include nucleic acids (e.g. for hybridization
assays), antibodies, DNA binding proteins, receptor pro-
teins or receptor binding domains, avidin/biotin and
other binding proteins or binding agents. These binding
agents preferably have functional groups that allow the
covalent linkage of the binding agent to the surface of a
solid substrate. Alternatively the binding agent may be
expressed on the surface of a cell.

An optically detectable labeling agent is also
required for the present assay. The macroparticle signal
is detected as a localized concentration of the optically
detectable label. The optically detectable label may be
present on the bead (as in Fig. 1A, 1D), on a binding
agent in solution (as in Figs. 1B-1C), or on the antigen.

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The optically detectable label, such as a dye, may be
attached to a bead. The dye may be tagged onto the bead
during manufacturing or bound to the bead by a surface
reaction. For example, beads functionalized with an
5   amino or carboxyl group can be conjugated to variety of
dyes such as cyanine dyes (e.g. Cy5, and Cy5.5), APC;
(Becton Dickinson BioSciences, San Jose, CA),
fluorescein, Texas red, rhodamine, FITC, PE, and numerous
other dyes. Conjugation to a functional group, such as
10   amino or carboxyl group, may be used to attach a dye to
the binding agent (such as an antibody) or to the anti-
gen. If the optically detectable label is a fluorescent
dye, it may be preferred for the dye to have an excita-
tion and emission wavelength above 590. Various sub-
15   stances present in biological samples may produce
autofluorescence when excited by wavelengths below 590
nm. The use of a dye that has an excitation and emission
wavelength above 590 nm, such as Cy5, Cy5.5, APC, Nile
Blue or FD&C Blue #1, is preferred for the assay of blood
20   samples. Components of blood (e.g. hemoglobin) auto-
fluoresce when excited by light below 590 nm. Methods
for limiting autofluorescence are further described in
U.S. Pat. No. 5,585,246, hereby expressly incorporated by
reference herein. Alternatively, the optically detect-
25   able compound may be chemiluminescent.

        Bacteria or other microbes may be detected
using the aggregation assay of the present invention. A
bacterial cell assay would be similar to the assays in
Figs. 1A-1F. The average size of bacteria is 0.2 μ,
30   roughly the same size as the preferred particulate sub-
strate. A typical microbial cell has numerous binding
sites on the cell surface. An assay could be developed
in which the binding agent binds to binding sites on the
microbial cell surface. As in the previous, the binding
35   agent or other reaction reagent is labeled with an opti-

cally detectable label. This agent could be combined in an assay mixture with a liquid sample that may contain microbes. If microbes are present, the bacteria would bind to the binding agent and aggregate, forming detectable macroparticles. If no bacteria are present, the unbound binding agent would remain as a background optical signal. In an alternative embodiment of the assay, the binding agent may be attached to a fluorescent bead. This bead may be from 0.05 μ to 10 μ in diameter, more preferably 2 μ. As in the previous references, the aggregated macroparticles could be gated by particle size and signal intensity.

In reference to Fig. 2A, bacteria 13 are shown having surface binding sites 15. When combined with a binding agent 17, the binding agent 17 will bind to the binding site 15 on the bacteria 13. Each binding agent 17 has multiple binding domains which may bind to multiple binding sites 15. If a number of cells are joined, an aggregated macroparticle 25 forms. The macroparticle is detected by size and/or signal intensity from the localized concentration of the optically detectable label 19 associated with the binding agent.

In contrast, Fig. 2B illustrates the assay in which no or very few bacteria are present. In this assay mixture, the bacteria 13 having binding sites 15 bind to binding agent 17 at binding site 15. Because few bacterial cells are present, additional binding agent domains 21 will attach to the binding sites 15 on bacteria 13. As a result, multiple microbial cells do not aggregate, and no microparticle forms. If no macroparticle signal is detected, microbial cells are not present.

The binding agent may, for example, be an antibody with a conjugated fluorescent label. The technology is adaptable for detecting large classes of microbes in a sample. For example, an antibody that binds to

peptidoglycan could be used as a universal bacterial binding agent. Peptidoglycan is a structural protein found in nearly all true bacteria. This antibody is further described in U.S. Pat. No. 4,596,769, hereby

5   expressly incorporated by reference herein. The use of this antibody allows broad spectrum bacterial detection. A more specific assay could also be developed using a binding agent specific to only certain species of bacteria.

10   The binding agent may be used as a reagent in solution in an assay mixture or may be conjugated to fluorescent bead. As in other applications, beads 0.05 μ to 10 μ may be used, with 0.2 μ beads preferred. The use of beads provides a larger macroparticle, which may be

15   preferable in some applications or with certain optical detection systems.

            The same technique may be applied for the detection of viruses, viroids, and virus-like organisms. Viruses are generally much smaller than bacteria (e.g.

20   Tobacco mosaic virus size: 3000 Å long x 180 Å diameter; mass = 40,000 Kd). Detection of these organisms would generally require a bead or other particulate substrate to act as a solid support for the binding agent to which the viruses bind.

25   In the prior art, agglutination assays required relatively high amounts of reagents, assay volumes, and particle counts. These assays were typically analyzed by turbidity or light scattering measurement or by direct visual examination. This generally requires analysis of

30   at least milliliter quantity of assay mixture containing high concentrations of beads or cells.

            In contrast, the present invention, through sensitive optical detection of individual macroparticles, enables sensitive assay of aggregation using microliter

35   quantity assay volumes and using bead concentrations

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which are at least 1,000 times more dilute than concen-
trations used in standard turbidity agglutination.

        The sensitivity of the present method may be
adjusted to modify the assay for a selected application.

5   By limiting the concentration of binding agent and/or
ligand (antigen), (e.g. concentration free in solution,
concentration on beads or cells, etc.) sensitive assays
could be developed. Optical detection using fluorescence
and chemiluminescence increases sensitivity several fold

10 over the usual turbidity measurement of agglutination.

        Conversely, increasing ligand intensity on a
fluorescent bead will decrease the sensitivity of an
aggregation assay. Optimization of concentrations of
binding and ligand reagents are important to modify the

15 assay sensitivity. Such modification allows a "tuneable"
sensitivity whereby the sensitivity of the assay may be
modified to fit a selected application.

    II.   Description of An Optical Detection System

20   As noted in the description of the assay, the
optical detection system must be able to detect and enu-
merate macroparticles by the detection of macroparticle
size and measurement of an optical label associated with
the macroparticle. One type of suitable optical detec-

25 tion system confines the detection to a limited depth of
field within an analytical container. The macroparticles
will settle within the limited depth of field and be
scanned by a focused light source. The optical detection
system has a light source that is selected to provide

30 light of a wavelength matched to the excitation or acti-
vation wavelength required for the optically detectable
label. In addition, the system has a detector that col-
lects light of the wavelength produced by the optically
detectable label. This system also measures signal pro-

35 file, which indicates macroparticle size. Various opti-

cal detection systems are compatible with these criteria.
One such system is the IMAGN 2000 sold by Biometric Imag-
ing (Mountain View, CA).

5   In Fig. 3, an optical system for limiting the
depth of field is illustrated. In this system a laser 10
directs laser light 80 onto a beam splitter 14. The beam
splitter is configured to direct light of the wavelength
of laser light 80 onto objective lens 19. Lens 19 fo-
cuses laser light 80 into a Gaussian waist 33. The
10   Gaussian waist 33 intersects the container 40 at the beam
waist having a width w. The area is thus provided with
the highest energy illumination.

The assay mixture is contained within container
40. The wavelength of laser light 80 is selected to
15   excite fluorescence from the optically detectable label
used in the assay. Excitation by laser light 80 produces
emission light 83 from target fluorescent compounds in
the assay mixture. This emitted light is gathered by
lens 19, which functions as a wide angle light collector.
20   The emission light falling on lens 19 from the illumi-
nated area is collimated by lens 19. This collimated
emission light 83 passes through beam splitter 14, which
is selected to allow light of the wavelength of emission
light 83 to be transmitted. The collimated light is
25   directed by steering mirror 22 onto focus lens 23. Lens
23 focuses the light through the aperture in spatial
filter 24 onto detector optics 31. Detector optics 31
may be configured to split the focused emission beam and
have distinct spectral ranges detected by separate detec-
30   tors.

The use of multiple detectors creates separate
detection channels to detect light above and below a
cutoff wavelength. This would allow detection of two or
more dyes at the same time if each dye has a specific
35   emission profile related to the selected wavelength.

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By matching objective lens 19 to spatial filter
24, the depth of field is confined to depth d. The posi-
tioning and optical properties of objective lens 19 will
determine the size and location of the Gaussian waist of
5   the excitation beam 80. Outside of the waist location,
the energy of the wavefront of the excitation light rap-
idly falls off. In addition, the position of lens 19
will determine the area from which light is collected.
Excitation light which originates beyond the depth of
10   field will be less likely to be collimated by lens 19.
The size of the aperture of spatial filter 24 is matched
to objective lens 19 to function as a stop for light
originating outside of the selected depth, limiting the
depth of field of detection to depth d.

15   The focused excitation beam is scanned by the
optical detector in a raster line scan of a depth within
the assay container. An optical oscillator scans the
excitation beam in a first direction while keeping the
waist in register within the analytical container. The
20   container is incrementally moved to provide for movement
of the beam in relation to the container in a second
direction. This allows optical interrogation of a volume
defined by the depth of field and the two dimensional
scan area. Additional description of the elements of
25   this system are found in U.S. Pat. No. 5,547,849 hereby
expressly incorporated by reference herein.

The detector measures the emission light inten-
sity, with signal capture recording measurements from
overlapping beam spot widths. Each captured measurement
30   is pressed and stored as a pixel within a plot. Inten-
sity measurement over a baseline intensity (average light
intensity measurement) are recognized as potential detec-
tion events. The pixels surrounding a detection event
are stored. The pattern of pixels in a neighborhood
35   surrounding a detection event are indicative of the size

of the particle detected.   The intensity of the detection
event indicates the amount of detectable label present at
a scan location.   This detection method is further de-
scribed in U.S. Pat. No. 5,556,764 hereby expressly in-
5   corporated by reference herein.

The optical detection system shown in Fig. 3
focuses light into the bottom of a container, such as a
microplate well.   The use of this system orientation
provides an advantageous analytical platform for screen-
10   ing a number of compounds for binding inhibition, binding
to a substrate, or other screening assays.   One such
system for scanning analysis of microplates is fMAT™
available from Perkin Elmer, Foster City, CA.   If the
optical system analyzes a container deeper than the depth
15   of field of detection, some macroparticle settling time
would be required to allow the macroparticles to settle
into the detection depth of field.   A five to ten minute
settling time should be sufficient to allow between 75
and 90% of the macroparticles to settle into the optical
20   depth of field in a microplate well.

Macroparticles could also be detected by a flow
cytometry system such as FACScan (Becton Dickinson, San
Jose, CA).   This system passes a stream of the assay
mixture past a focused light source.   Particles in the
25   stream scatter light which is detected by sensors.   Emit-
ted light may also be detected by sensors.   The scatter-
ing measurement indicates particle size while fluorescent
intensity indicates amount of optically detectable dye
that is associated with the macroparticle.

30   Fig. 4 provides an illustration of the detec-
tion of microparticles and macroparticles in an assay
mixture.   In assay mixture 90, some fluorescent reagents
91 are in solution, not bound to any associated target.
These unbound reagents produce a background optical sig-
35   nal 112.   Also in the assay mixtures are a number of

microparticles 93 that will produce a microparticle sig-
nal 114 which may be detectable over the background sig-
nal 112.   The microparticle signal is a localized concen-
tration of the detectable label either bound to the bead

5   or associated with the bead.   The macroparticle 95 gives
a macroparticle signal intensity 114 that is detectably
greater than the microparticle signal intensity. This
results from the macroparticle having a greater localized
concentration of the detectable signal than the

10   microparticle.   In addition to signal intensity, the
signal profile of the macroparticle detection signal 116
is detectably different than the signal profile of the
microparticle detection signal 114.   The signal intensity
and two dimensional profile may be used alone or in com-

15   bination to detect microparticles and macroparticles.
Detection is most robust if the size of the macroparticle
is at least 50% of the beam spot size (size of focused
beam waist).

20   III.   Examples
The preceding generalized description is fur-
ther illustrated with the following examples.

Example A.   Detection of digoxin using an aggregation

25   assay
Digoxin is a small molecule pharmaceutical
compound with a molecular weight of 781 used to treat
heart conditions.   Monitoring digoxin levels in a patient
could be important in determining efficacy of cardiac

30   treatment.   Additionally, competitive binding assays
containing soluble digoxin could serve as an initial
indication of compound binding to cardiac receptors.
This information would be useful in evaluation of a pro-
spective cardiac therapeutic compound.

35

Aggregation assay of hapten or small molecule

1.   Conjugation of digoxigenin to Bovine Serum Albumin:

Digoxin can be conjugated to BSA by following
the periodate and other procedures (Butler, V.P. and
Chen, J.P. Digoxin specific antibodies. PNAS 57:71-78
(1967); Tijssen, P. in PRACTICE AND THEORY OF ENZYME
IMMUNOASSAY, pp. 279-296, 1985).   Step-by-step procedure
for preparing fluorescent Blue bead (Bangs Lab, Fishers,
IN) digoxigenin conjugate is as follows:

First, digoxigenin (hapten) was conjugated to
bovine serum albumin (BSA).   Digoxigenin which has simi-
lar cross-reactivity to digoxin is used in place of
digoxin.   Digoxigenin can be functionalized with a car-
boxyl group (Brinkley, M. A brief survey of methods for
preparing protein conjugates with dyes, haptens and
cross-linking reagents.   Bioconjugate Chemistry 3:1-13,
(1992)).   Once the digoxigenin hapten has been prepared
containing the carboxyl group, it can now be conjugated
to BSA as follows.

10 mg of digoxigenin hapten was placed in a 5
ml glass vial.   1.2 M excess each of N-hydroxysuccinimide
(NHS) (3.2 mg) and 1-ethyl-3-(-dimethylamino-
propyl)carbidiimide HCl (EDAC) (5.3 mg) were added to the
glass vial containing the digoxigenin-COOH.   The mixture
was dried for 5 hours in vacuum in a desiccator.   The
vial was removed from the vacuum and opened under nitro-
gen.   The vial was immediately capped with a rubber sep-
tum.   1 ml of HPLC grade dimethylformamide (DMF) was
added to the vial through the septum using a syringe.
The mixture was stirred overnight at 4°C, forming the
activated digoxigenin hapten.

Activated digoxigenin hapten was added to BSA
to prepare hapten BSA conjugate.   BSA was dissolved in 50
mM Tris buffer, pH 8.0 at 10 mg/ml.   Activated
digoxigenin hapten was placed in a 1-ml glass syringe

suitable for using with a syringe pump. The syringe pump
device was set up at room temperature and set at 1
ml/hour addition. For 1 mole of BSA, 85 mole of acti-
vated digoxigenin hapten was added to produce the conju-
5   gate as follows: One ml BSA solution was placed on a
magnetic stirrer to stir the solution. Activated
digoxigenin hapten was added to the BSA solution at the 1
ml/hour rate specified above. This mixture was continu-
ously stirred while the activated digoxigenin hapten was
10   added. Once all of the activated digoxigenin was added,
the mixture was stirred for an additional hour.

          Following the combination of BSA to the acti-
vated digoxigenin hapten, the conjugate mixture was dia-
lyzed over running tap water for 1 week, followed by
15   purification on Sephadex G50 column in 25 mM 2-(N-
morpholino)ethanesulfonic acid (MES) buffer, pH 6.1. The
protein in the conjugate was determined by Pierce BCA
protein assay (P/N 23225, Pierce, Rockford, IL). The
amount of digoxigenin conjugated to BSA was determined by
20   differential absorption spectrophotometry.

2. Conjugation of BSA-digoxigenin to Fluorescent Blue
    Bead

          Fluorescent beads were used in this experiment.
25   These beads are available with various types of func-
tional group on them such as amino (NH_2) group (P/N 2600,
size 0.06 µ), carboxyl (-COOH) group (P/N 4169, size 0.19
µ) from Bangs Lab (Fishers, IN). Similar beads are
available from other commercial suppliers [e.g. Sky Blue
30   bead from Spherotech, Libertyville, IL].

          The instant assay used -COOH Blue bead (0.19 µ)
and covalently attached to the -NH_2 group on BSA of BSA-
digoxigenin conjugate. Adsorption of the BSA digoxigenin
conjugate protein precedes the covalent coupling. There-
35   fore, a protein binding isotherm must be first estab-

lished to determine the appropriate concentration of BSA-
digoxigenin to be conjugated to the -COOH Blue bead. A
100 µg protein (BSA-digoxigenin)/mg bead is preferred.

Set up the binding reaction by pipetting the fol-
lowing in a tube:

50 µL of 500 mM MES stock buffer (MES=(2-(N-
morpholino)ethan sulfonic acid, pH 6.1): 25 mM final
concentration.

100 µL of 1% solid stock bead particles: (0.1%
solids final volume)

1 mg BSA-Digoxigenin based on 280 µm protein deter-
mination ($\simeq$ 15.4 µM)

Water to make 1 ml final volume

These compounds are mixed in the tube for 30
minutes on a mixing wheel at room temperature.

Calculate the EDAC amount and use it in 2.5-
fold molar excess over the BSA carboxyl group (0.011 mg
in this example).

The EDAC solution is prepared just before use
and the calculated amount is mixed rapidly into the
reaction by mixing repeatedly with the pipettor.

Following addition of EDAC, the tube is mixed
at room temperature on a mixing wheel for 1 hour. After
the mixing, the unbound protein is removed by 5 times
centrifuging the tube at 20,000 g for 20 min and decant-
ing the supernatant. The pellets are resuspended to 0.1%
solids in the 25 mM MES buffer by probe ultrasonication.

Finally the BCA protein assay is run to deter-
mine the protein conjugated to the bead. The reading of
the supernatant is made after centrifuging the protein
assay tubes to remove particles (Ref: Micro reagent
Optimization Manual; Seradyne, Indianapolis, IN).

3.   Preparation of Digoxigen Standard

           A digoxin stock solution is prepared at 1 mg/ml
in DMF. Dilute in 50 mM -[2-hydroxyethylpiperazine-N'-[2-
ethanesulfonic acid] or HEPES, pH 7.2 to give final con-
centration in ng/ml: 0, 1, 2, 3, 5, 10.

4.   Preparation of antibody reagent

           Digoxigenin specific antibody can be produced
by using methods known to those of skill in the art.
Many antibodies are commercially available.   Prepare
appropriate antibody dilution in borate buffer.

5.   Assay protocol

           Both bead and antibody reagents are optimized
to give maximum signal in assay containing no test sam-
ple.   Optimization protocols are similar to those used in
example E.   The zero sample will give maximum relative
cell intensity (RCI).   The RCI will decrease proportion-
ately with increase of sample in the reaction mixture.   A
representative protocol is given below:

           7 tubes containing the assay mixture are assem-
bled: six for the digoxin standards at 0, 1, 2, 3, 5, and
10 µg/ml respectively, and one tube for the unknown
sample.   20 µL of standard or sample is added to each
tube.   100 µL of Bead reagent is added to each tube.
Bead reagent and antibody reagent are added at an opti-
mized level as determined by the protocol mentioned in
Example E.   The final volume in each tube was made to 1
ml with borate buffer.   The tubes are vortexed and subse-
quently incubated for 1 h. 50 µL of the solution from
each tube is then added into a VC120 capillary sold by
Biometric Imaging (Mountain View, CA).   The sample is
then analyzed on an IMAGN 2000™.   The results may be used
to plot RCI vs. digoxin concentration for the 6 (six)
digoxin standards.   The concentration in the unknown test

sample can then be determined from the graph by correlat-
ing the intensity from the test sample to a position on
the graph.

5   Example B.   Detection of Alpha-fetoprotein in an
                    aggregation assay
            Alpha-fetoprotein is a large molecule
       (MW=70,000) glycoprotein used as a marker for prenatal
       detection of fetal anomalies and certain types of cancer
10   in non-pregnant patients. The carbohydrate portion of
       the molecule has a vicinal -OH group which can be con-
       verted to aldehyde group by periodate oxidation. This
       aldehyde group will react with an amino group to form
       Schiff's base. Therefore, for this application, bead
15   with amino functional group is used. The alpha-
       fetoprotein is conjugated to an amino functionalized
       fluorescent bead via the aldehyde group in methods simi-
       lar to those used in example A.
            It is not necessary to use only particles hav-
20   ing carboxyl or amino groups. The alpha-fetoprotein
       could be adsorbed directly on to the particle surface.
       However, the scheme described above is preferred to ad-
       sorption because covalent bonding produces a more robust
       conjugate compared to adsorption. Protocol and optimiza-
25   tion remain the same as given in example E.
            Depending on the use, either a quantitative or
       threshold determining assay can be developed. Alfa-
       fetoprotein (AFP) levels greater than 1000 µg/L may be
       indicative of some types of cancer (except during preg-
30   nancy). For a threshold determining assay, one standard-
       ized level of AFP would be run with samples and the RCI
       of the standard or test sample compared. For quantita-
       tive assays, a standard curve can be prepared as dis-
       cussed for digoxin. Procedures for reagent preparation
35   and optimization remain the same as discussed for

streptavidin in example E. Many variations of this tech-
nology are possible as shown in Figs. 1A-1F.

Example C.   Avidin/Biotin Macroparticle Formation

5   In a third example, biotin is used as a binding
agent to cause aggregation of avidin coated particles.
Biotin is a 244 dalton molecule found in blood and tis-
sue. It has a very high affinity for avidin/strept-
avidin. Avidin is a tetrameric glycoprotein with a mo-

10   lecular weight of approximately 66,000. It is primarily
derived from egg whites. Like avidin, streptavidin is a
tetrameric protein with similar molecular weight and
binding characteristics. Streptavidin is isolated from
Streptomyces avidini. Streptavidin has advantages in certain

15   applications in comparison to avidin because avidin has
differing protein glycosylation. Avidin may be subject
to non-specific binding in certain application because of
its associated carbohydrates.
               In the present example, avidin-biotin binding

20   is used in the agglutination of beads. This agglutina-
tion is subsequently detected on an optical detection
system in a limited depth of field. In this assay, a
conjugate of streptavidin-Cy5 is utilized. Biotin coated
beads (biotin-bead 6 μ in size) are used. These beads

25   are non-fluorescent alone, but become fluorescent after
streptavidin-Cy5 binds to biotin, which is covalently
attached to the bead. As noted earlier, such an assay
alternatively could include fluorescent labeled bead
instead of a fluorescent reagent.

30   The optical system used to analyze the assay
mixture was the IMAGN 2000™ (Biometric Imaging, Mountain
View, CA). This system has an optical configuration
similar to that shown in Fig. 3. A laser produces a beam
which is focused by a lens into a container. The focused

35   light excites fluorescence which returns in a retrobeam

to a detector. The focusing lens also acts as a light
collector. A spatial filter, acting in combination with
the light collector, limits the depth of field from which
light is collected. In general, this limited depth of
5   field is from 25 to 250 microns. The IMAGN 2000 system
scans from the top down into a container, an opposite
optical configuration of that shown in Fig. 3. This
system is generally used to scan rectangular capillaries
having a limited fluid depth. Thus the capillary depth
10   may structurally limit the scanned depth and act in con-
junction with the optical system to limit the depth of
field. As noted in relation to Fig. 4, such a system is
able to detect discrete events which are measured by
detection of intensity of the retrobeam over a selected
15   threshold. The scanner analyzes a specific volume of
fluid defined by the scan width and length and the opti-
cal depth of field. The number of detection events may
be tabulated as detection events per unit volume (e.g.
cells/µL, beads/µL or macroparticles/µL). The optical
20   system detects the relative cell intensity (RCI) which is
a measurement of the average intensity of detection
events. The peaks are detected from macroparticles,
cells, or beads. In addition the system measures back-
ground limiting intensity (BLI), which is a measurement
25   of the average intensity of the background wavelength.
By scanning in a limited depth of field, an assay mixture
may be analyzed without removing unbound optically la-
beled reagents. The relatively high signal from targets
with higher localized concentration of associated optical
30   dye allows detection in the presence of unbound dye.
This enables homogenous assays, i.e. assays that do not
require a separation step. In addition to RCI, the sys-
tem also detects emission signal profile, allowing size
gating. This optical analytical system may be used to
35   analyze detection assay mixtures for detecting small

molecules, large molecules, insoluble antigens, and a
variety of cells. It is anticipated that any optical
system which provides detection in a limited depth of
field and is capable of measuring optical signals may be
5   adaptable to the present assays.

In this example, amino beads (Becton Dickinson
Biosciences, San Jose, CA) were used. Biotin was then
conjugated to the amino group on the bead (Stock 200,000
beads/μl). The biotin coated beads were diluted with
10   borate buffer (100 mM borate buffer contains 0.1% bovine
serum albumin, 0.05% Tween 20, 100 mM EDTA and 0.05%
azide) to give a final bead number per unit volume (200-
1000 beads per μl). Concentration of avidin/streptavidin
that will give a useful dynamic range in the assay was
15   determined experimentally as illustrated in the next
example. All dilutions were made in borate buffer unless
otherwise specified.

A number of assay mixtures were assembled, each
containing a standard amount of the suspended biotin
20   beads, streptavidin-Cy5 (FluoroLink™ Cy™5 Amersham
Pharmacia Biotech, conjugated to streptavidin) in borate
buffer to a total volume of 1 ml. A fixed aliquot of
biotin beads were added initially to a 1 ml eppendorf
tube. A varying amount of avidin was added to each tube,
25   that was subsequently mixed by vortexing. Following
vortexing the tubes were incubated. Following incuba-
tion, a fixed amount of streptavidin conjugate was added
to each tube, vortexed and incubated. Borate buffer was
added to each tube to achieve a final volume of 1 ml.
30   The tube was vortexed to mix the contents of the tube.
50 μL of the mixture was loaded into each of two VC120
capillaries (Biometric Imaging, Mountain View, CA) and
placed on a VC2 cartridge (Biometric Imaging, Mountain
View, CA). The cartridge was loaded on the IMAGN™ 2000
35   instrument and analyzed using CD34 assay software (Bio-

metric Imaging, Mountain View, CA). Initial experiments
indicated that signal intensity of produced
macroparticles was very intense. To compensate the in-
strument photomultiplier tube (PMT), gain was reduced by

5   50%. Other gating parameters were unchanged from those
specified for the CD34 assay.

Table 1 - Experiment Protocol

Cartridge Set	1	2	3	4	5	6
µL Avidin (5 mg/ml), µL	0	5	10	20	50	100
Borate buffer, pH 8.6, µL	100	95	90	80	50	0
µL Steptaviden -Cy5 conju-gate, **	10	10	10	10	10	10

Mix contents by vortexing

** Steptaviden-Cy5 stock 1 mg/ml diluted to 1000x. 10 ng SA-Cy5 in 10
µL volume

Biotin Bead (20x dil.), µL	20	20	20	20	20	20

Mix contents by vortexing
Incubate at RT, 30 min, shaking, medium speed on American rotator.

Borate buffer, pH 8.6, µL	770	770	770	770	770	770
FD&C Blue #1, (1 mg/ml stock; 200x diluted) µL	100	100	100	100	100	100

Mix contents by vortexing
Load VC cartridge with VC120 capillary and read

Table 1 lists the assay mixture components for
each of six cartridges analyzed. As noted, each car-
tridge contains two capillary tubes containing 50 µL of

40   assay mixture. The streptavidin Cy5 conjugate is a 1
mg/ml streptavidin-Cy5 stock solution diluted 1000X in
borate buffer.

Table 2 - Avidin-Biotin Macroparticle Formation Assay

45   STELLer CD34 Assay, Version CD34-3.07B
GainO=84; Gain1=144 (50% less gain)

	Avidin μL added	Avidin μg/ml*	BLI A	RCI A
Cartridge 1	0	0	265	6879
Cartridge 2	5	25	272	668
Cartridge 3	10	50	272	486
Cartridge 4	20	100	283	312
Cartridge 5	50	250	288	259
Cartridge 6	100	500	289	179

* μg in 1 ml reaction mixture

The results of the analysis of the assay mix-
tures are listed in Table 2. In general, with increase
in avidin concentration, a decrease in RCI is observed.
The RCI provides a good correlation with the concentra-
tion of avidin at the current instrumentation parameters.

Dot plots at each concentration of avidin are
shown in Figs. 5A-5F. Fig. 5A is a dot plot showing
measurements of detection event intensity (MaMo) and size
(FitArea O), measuring the assay mixture containing 0 μL
avidin. In this reaction, streptavidin-Cy5 binds to the
biotin bead without competition from free avidin in solu-
tion, resulting in macroparticle aggregates ranging in
size up to index 4. This aggregate produces a very in-
tense optical signal, indicated in the dot plot by the
number of intensity events measured in the extreme right
of the intensity scale (X-axis). Fig. 5B illustrates the
measurements made of the assay mixture containing 5 μL
(25 μg) of avidin. This graph illustrates that the de-
tection events are significantly less intense than seen
in Fig. 5A. This trend continues in Figs. 5C, 5D, 5E,
and 5F. The reduction in signal intensity correlates to
the amount of free avidin in solution. Addition of in-
creasing amounts of avidin increases competition between
labeled streptavidin and free avidin for binding to bio-
tin on the biotin coated bead. When avidin binds to the
bead, less labeled streptavidin may bind, reducing the
detected signal. From these dot plots, it is apparent
that this assay allows very sensitive detection of
analyte in solution. The macroparticle size acts as a

-35-

second indicator of competition. Each of avidin and streptavidin has tetrameric binding sites and each promotes macroparticle formation. Since the concentration of streptavidin-Cy5 conjugate remains constant, the increasing concentration of avidin in the assay mixture participates in aggregation reaction and maintenance of the aggregate size. However, the aggregate moves to the left on the intensity scale with increasing avidin due to reduced label in the aggregate. This movement can be measured in RCI and is related to avidin concentration in the reaction mixture. This is observed in Figs. 5A-5F.

Example D.   Avidin-biotin macroparticle formation with
              detection using flow cytometry

In the next example, avidin-biotin binding and the formation of macroparticles are analyzed using IMAGN 2000 system and compared to analysis using a flow cytometer. In the assay mixture assembly, two types of beads are used. One bead is coated with biotin. The second bead is conjugated to streptavidin and is optically detectable through an associated blue label impregnated into the bead.

Avidin-Biotin System: Flow vs. Imagn

Table 3 - Experimental Protocol

Tube No.	Biotin bead	SA Blue bead dil.	SA Blue bead	SA-APC	Buffer
1	25	-	-	-	475
2	25	1000X	68	-	475
3	25	100X	68	-	475
4	25	10X	68	-	475
5	25	-	-	20	475

*   Tubes #2, 3, 4 have SA blue bead 360, 3600 & 36,000 beads per
     biotin bead, respectively.
*   Biotin bead diluted 10X (Stock: 200,000 beads/µl).

Note:

a. All additions are µL.
b. Biotin bead are 6 µM; 200000 bead/µL.
c. SA Blue bead (Bangs) are 0.19 µM; 2.628+e9 beads/µL.
d. SA = Streptavidin

The assembly of the reaction mixtures is described in conjunction with Table 3. In this method the biotin beads as used in Example C were diluted 10X with borate buffer. 25 µL of diluted beads was added to each

5   tube, as stated in Table 3. Streptavidin Blue bead was added as indicated in Table 3. The final volume in each tube was brought to 500 µL with borate buffer. The tubes were vortexed and incubated at room temperature for 15 minutes. The beads were subsequently washed two times by

10   adding 2 ml borate buffer for each wash, and centrifuging the contents at 5000 RPM in a Sorvall RT6000 centrifuge for 5 minutes. Liquid in the tubes was decanted to remove unbound streptavidin Blue bead. The remaining beads at the bottom of the tubes were suspended in the 0.5 ml

15   borate buffer and analyzed both on FACSCalibur™ (Becton Dickinson, San Jose, CA) and IMAGN 2000™ analysis system. The first reaction mixture does not contain any streptavidin Blue bead. The light scattering and fluorescent intensity measurements made on flow cytometry

20   instrumentation are shown in Fig. 6. The detected Biotin beads are in group R1 with very low non-specific aggregates in group R2. These particles are of relatively small size and relatively low fluorescent intensity, and this set serves as control for tubes 2, 3 and 4 (Table

25   3).

The second assay mixture has 360 streptavidin Blue bead per biotin bead. In flow cytometry analysis of this assay mixture, macroparticles containing two, three and four biotin beads are seen as indicated by the scat-

30   tering plot 7A and are indicated by squares R2, R3 and R4 respectively. The fluorescent intensity shows a similar grouping. In addition to the relatively low fluorescent intensity characteristic of group I (RI) positioned at 200 on the X axis of the fluorescent intensity graph,

35   additional groups are seen at 400, 600, and near 800. These three additional intensity groups correspond to scattering groups R2, R3 and R4. The analysis of the second reaction mixture was also performed on the IMAGN

2000 the results of which are shown in Fig. 8A. The
combined signal intensity and particle size are measured
by this system. These results in terms of size index
correspond well to the results determined by flow
cytometry. The size index (signal fit area) ranges from
0.5 to 4 in IMAGN dot plot (Fig. 8A) that indicates scat-
tering groups R2, R3 and R4 in flow scattergram (Fig.
7A).

The third assay mixture has 3600 streptavidin
Blue beads per biotin bead. Again the results are shown
for flow cytometry in Fig. 7B and for IMAGN 2000 in Fig.
8B. The flow cytometry results are similar to those for
Fig. 7A. Macroparticles of four different sizes were
detected. For the IMAGN 2000 detection, Fig. 8B corre-
sponds to the flow cytometry results with the
macroparticles detected having a signal fit area between
0.5 and 4.

The fourth assay mixture has 36,000
streptavidin Blue beads per biotin bead. At this high
streptavidin Blue bead concentration, the flow cytometry
plot shown in Fig. 7C indicates that groups R3 and R4
(detected larger macroparticle aggregates) have signifi-
cantly diminished. This is likely due to the increased
competition of the streptavidin beads for biotin bead
binding. Fig. 8C illustrates detection on the IMAGN 2000
system. The results correlate well with flow cytometry,
with particles detected ranging in size index (fit area)
from 0.5 to 3 with less number of bigger particles pres-
ent. Finally assay mixture 5 contains only streptavidin-
APC, 6 µ beads. Analysis of this assay mixture by flow
cytometry produces an intense measurement in the R1 group
(indicating 6 µ size as Biotin bead in Fig. 6) as shown
in Fig. 7D. This very intense reading was similarly
measured in the IMAGN 2000 as shown in Fig. 8D.

The flow cytometry results correlate well with
the IMAGN 2000 results. In both systems, the analysis
shows both aggregation, and rosetting are occurring de-
pending on the amount of streptavidin blue bead used in a

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sample. In assay mixtures containing very high concen-
trations of streptavidin Blue bead, additional rosetting
and less aggregation are seen in both flow cytometry
measurement and IMAGN 2000 plots; as seen in comparison
of assay mixture 2 (high aggregation as illustrated by
large macroparticle formation) and assay mixture 4 (rela-
tively low macroparticle size and decreasing intensity
indicating rosetting).

Example E.   Antibody/antigen system for macroparticle
             formation
        In the next example, an antibody is used as the
binding agent for the aggregation of particles.

Table 4. Aggregation Using Antibody/Antigen System
Experiment Protocol for Streptavidin (SA) Blue bead
and streptavidin antibody

Item descrip-tion	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5
Rabbit anti-SA antibody	100	100	100	100	100
SA Blue bead	100	50	20	10	0
Borate buffer	0	50	80	90	100

Incubate overnight shaking at room temperature on American
rotator.

Background dye (200Xdil)	100	100	100	100	100
Borate buffer	700	700	700	700	700

Mix and load VC120 capillary. Run STELLer CD34 assay.

SA = Streptavidin

Antibody was diluted 32X in borate buffer per manufacturer's
recommendation.

SA Blue bead was diluted 1000X in borate buffer. Aliquots
were added from this dilution as indicated above. The SA Blue
bead is supplied as 1%. After 1000X dilution, its conc. will
be 10 ng/µL.

Background dye is 1 mg/ml stock of FD&C#1 in water. All
dilutions made from this stock.

Assay mixtures were assembled each containing a
varying amount of the antigen coated beads. Table 4
describes the reagents combined to form each of the five
assay mixtures. The streptavidin antigen and rabbit
5   anti-streptavidin (anti-SA) antibody were obtained from
Sigma (St. Louis, MO). The antibody was diluted 32X in
borate buffer as per manufacturer's recommendation.
Streptavidin Blue beads were diluted 1000X in borate
buffer. The final concentration of the streptavidin Blue
10   beads after 1000X dilution will be 10 ng/μL. The back-
ground dye supplied is 1 mg/ml stock of FD&C Blue #1 in
water. All dilutions of background dye are made from
this stock. The five assay mixtures assembled contain a
fixed amount of the antibody reagent (100 μL) added into
15   each assay mixture. Varying amounts of streptavidin Blue
bead are added to each assay mixture. Borate buffer was
added to bring the mixture volumes to a common amount.
Following assembly, 50 μL of the assay mixture was added
to VC120 capillaries and analyzed using the STELLer CD34
20   software (gain 0 = 67, gain 1 =124; normal noise and size
gate) run on the IMAGN 2000™.

Aggregation Using Antibody/Antigen System

25   Table 5
Effect of varying concentration of streptavidin (SA) Blue particle on
streptavidin antibody

Assay Mixture	SA bead, μL added	Beads/μL	BLI	RCI	IMAGN error messages
5	0	2.5	401	860	value outside range
4	10	18.5	384	988	–
3	20	73.5	394	1991	–
2	50	293	427	3819	–
1	100	274.7	427	3118	too many events

40   Table 5 and Figs. 9A-9E set up the results of
this analysis. An assay mixture 1 using 100 microliters
of streptavidin blue beads resulted in graph 9A in which
the detection events were too numerous for the optical
analytical system to distinguish as indicated by error
45   message. In the second, third and fourth reaction mix-
tures, the relative cell intensity and background light

index are within the detectable ranges. Figs. 9B, 9C and
9D illustrate the dot plots of the cellular detection
events. In assay mixture 5 (Fig. 9E), no streptavidin
Blue beads were present and very few detection events
5   were measured. With respect to Table 5, increasing con-
centration of streptavidin Blue bead and the reaction
mixture corresponds with increasing amounts of Blue bead
which are being bound to the anti-streptavidin antibody.
The numbers of beads/µL and relative cell intensity are
10   decreasing with decreasing addition of SA-Blue bead to
the reaction mixture as shown in plots 9A-9E. The back-
ground light intensity remains similar at all concentra-
tions as expected. With no beads present in the reaction
mixture, fluorescence from beads will not be generated
15   and the instrument will detect no beads. With increasing
concentrations of beads, the aggregation into
macroparticles will occur and the instrument will detect
aggregated macroparticles as events. These events will
be enumerated and the intensity of the events measured.
20   The minimum size index threshold for this experiment was
set to 1.25. This setting gates out non-aggregated
beads, targeting detection of only macroparticles.

            The gating out of non-aggregated beads is shown
in dot plots 9A-9E generated by the IMAGN 2000. In assay
25   mixture 5 containing no streptavidin Blue beads; only the
background noise is present as events below the selected
size index (Fig. 9E). At 10 µL bead mixture used in
assay mixture 4, the agglutination to form macroparticles
is seen although most of the events have detection at
30   relatively low intensity and the size of detection events
is concentrated near the 1.25 gating threshold, with a
few macroparticles ranging from 1.5 to 4, as shown in
Fig. 9D. Fig. 9C illustrates the dot plot of the analy-
sis of assay mixture 3 containing 20 µL streptavidin Blue
35   bead. This plot shows many additional detection events
above the 1.25 gating threshold with many additional
detection events ranging from 1.5 to 4. This trend con-
tinues in the second and first assay mixtures, the detec-

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tion results of which are illustrated in Figs. 9B, 9A
respectively. It appears that in this assay
macroparticles aggregate to a maximum size index of 4.
With additional streptavidin Blue bead concentration,
additional aggregated macroparticles are produced, with
additional beads also correlating to higher macroparticle
intensity. Excluding intensity measurement, the maximum
number of large size macroparticles are formed when the
bead concentration is between 20 and 50 µL in the assay
mixture. In this initial assay, the optimized amount of
streptavidin Blue bead concentration in a streptavidin
detecting assay is determined. When excess Blue beads
are used, the instrument is unable to detect discrete
events resulting in error signal "too many events de-
tected". This results from an excess of non-aggregating
beads providing an optical signal in excess of the abil-
ity of the optical system to detect and process.

Table 6 - Experiment Protocol

Cartridge Set	1	2	3	4	5	6
*SA --> ng	0	125	250	500	1250	2500
Free SA (25 µg/ml)	0	5	10	20	50	100
Borate buffer	100	95	90	80	50	0
SA-Blue bead (1000x dil)	25	25	25	25	25	25
Rabbit anti-SA antibody	100	100	100	100	100	100

*ng added in 1 ml reaction mixture.

Incubate over the week end at 4C and then 1h shaking at RT

Borate buffer*	675	675	675	675	675	675
Background dye (200x dil)	100	100	100	100	100	100

Mix and load VC120 capillary. Run STELLer CD34 assay.

*After adding borate buffer, 0.4 ml was taken out and 40 µL of
200x diluted dye was added.

SA stock of 25 µg/ml used in this experiment

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Background dye is 1 mg/ml stock of FD&C#1 in water. All
dilutions made from this stock.
All additions are in µL.

5

        The experiments described in conjunction with
Table 4 and Table 5 provided a concentration of
streptavidin Blue bead which would provide a sensitive
assay for streptavidin detection via aggregation. Table

10   6 outlines the experimental protocol in assembling the
assay mixture. As was determined in the previous set of
experiments, 25 µL streptavidin Blue bead was prepared in
borate buffer and used in the assay. A rabbit anti-
streptavidin antibody was used with 100 µL added per

15   reaction mixture. Amounts of free streptavidin varying
from 0 µL of free streptavidin to 100 µL of free
streptavidin was added to each assay mixture. Other
remaining components of the reaction mixtures were iden-
tical to those in the previous assay mixture (Table 4).

20   The noise gating in the STELLer CD34 software was set to
10 to avoid "too many events detected" error message on
the IMAGN 2000 instruments.
        In this group of assay mixtures, both free
streptavidin and streptavidin Blue bead will bind to the

25   anti-streptavidin antibody. Either binding agent will
effect formation of macroparticles. If most of the anti-
body binding sites are occupied by free streptavidin, the
relative cell intensity signal detected by the optical
detection method will be weak. In contrast, if the ma-

30   jority of the sites are bound by streptavidin Blue
beads, the macroparticles detected will have relatively
high signal intensity.

Table 7

**Effect of free streptavidin on aggregation:
Antibody-Antigen system**

SA, µL	BLI A	RCI A
0	293	3202
5	288	1103
10	274	879
20	319	802
50	273	828
100	283	516

Table 7 shows the data from an IMAGN 2000 analysis of the six assay mixtures. These results are also shown in Figs. 10A-10F. Data in Table 8 demonstrate that there is a linear relationship between the free streptavidin (between 20 µL to 100 µL streptavidin) and RCI in the assay mixture. The RCI decreases with increasing free streptavidin concentration. Fig. 10A shows that when no free streptavidin is included in the assay mixture, only streptavidin Blue beads bind to the anti-streptavidin antibody resulting in very high RCI of 3202. The assay mixtures containing 5 µL, 10 µL and 20 µL free streptavidin in the assay mixture, when analyzed, show macroparticle formation with increasing macroparticle size index and corresponding decrease in relative cell intensity. This is shown in Figs. 10B, 10C and 10D. In assay mixtures with 50 µL and 100 µL, free streptavidin in the assay mixture, the relative cell intensity has decreased significantly. This is shown in Figs. 10E, 10F. Because of the dim signal, some events may not be detected by the instrument. It is thus difficult to assess the size of the aggregated macroparticles in the presence of high concentration of free streptavidin. However, within the range in assay mixtures 1, 2, 3 and 4 sensitive detection of the free antigen through measurement of both size of macroparticle and intensity of macroparticle signal is achieved.

As the foregoing examples illustrate, the present assay provides a highly sensitive assay for the detection of haptens or large molecules. The assay can be used for detection of a target compound in solution, the assay of binding to a compound, or a competitive binding assay for screening. A number of different optical systems may be used to analyze the assay mixtures and detect macroparticle optical signals.

Claims

1.   A method for the detection of individual aggregated macroparticles, the method comprised of the steps:
5   combining in an assay mixture a particulate substrate or cell, said particulate substrate or cell associated with an antigen binding site or binding agent, with an associated assay solution wherein either the binding agent, or ligand, or the particulate substrate or
10   cell has an associated optically detectable label, wherein under binding conditions the particulate substrate or cell may aggregate to form a macroparticle;
             directing a beam to optically interrogate the assay mixture, the interaction of the beam and the
15   macroparticle producing a macroparticle optical signal event wherein the assay solution is scanned such that individual macroparticles are detected; and
             detecting optical signal events wherein said macroparticle optical signal event is distinguishable
20   from optically detectable label not associated with a macroparticle, thereby measuring the agglutination in the assay mixture.

2.   The method of claim 1, wherein the step of directing
25   a beam to optically interrogate the assay mixture is effected by,
             adding the assay mixture to an analytical container;
             scanning a focused beam spot in two dimensions
30   through the assay mixture in said container;
             measuring the light intensity emitted from a limited depth of field within the scanned depth; and
             determining if localized light intensity measurement are above a background intensity.

3.   The method of claim 2, wherein directing the beam
spot includes directing a focused beam spot having a beam
spot size selected so that the non-aggregated cells or
particulate substrates have a width which is 0.5 to 10
5   times the width of the beam spot.

4.   The method of claim 1, wherein the step of detecting
optical signal events includes at least one step selected
from the steps of detecting optical signal intensity and
10   detecting optical signal profile.

5.   The method of claim 1, wherein the step of
contacting a particulate substrate or cell with a binding
agent is effected by contacting a bead coated with
15   surface receptors with a binding agent.

6.   A system to measure aggregation, the system
comprising:
            an assay container;
20   an assay mixture contained within the assay
container, the assay mixture including,
                a) at least one microparticle,
                b) at least one binding agent,
                c) at least one optically detectable
25   label,
                d) at least one binding site wherein the
microparticle is conjugated to one of the group
consisting of the binding agent and the binding site such
that under binding conditions microparticles aggregate to
30   form macroparticles which settle to a specific depth of
the container, said macroparticles at a concentration
within the container such that the macroparticles are in
a non-overlapping, detectably spaced orientation within
the analytical container; and

an optical detection system configured to
optically interrogate the container in a limited depth of
field, the depth of field containing the macroparticles,
said optical system distinguishing macroparticles from
5   microparticles in a homogenous assay mixture.

7.   The assay system of claim 6, wherein the container
is a capillary.

10   8.   The assay system of claim 6, wherein the micro-
particle is a bead.

9.   The assay system of claim 8, wherein the bead is
labeled with the optical label.
15

10.   The assay system of claim 6, wherein the optically
detectable label is selected from the group comprising a
fluorescent dye and a chemiluminescent agent.

20   11.   The assay system of claim 6, wherein the optical
system is a laser scanning system.

12.   The assay system of claim 6, wherein the binding
site is present on an antigen.
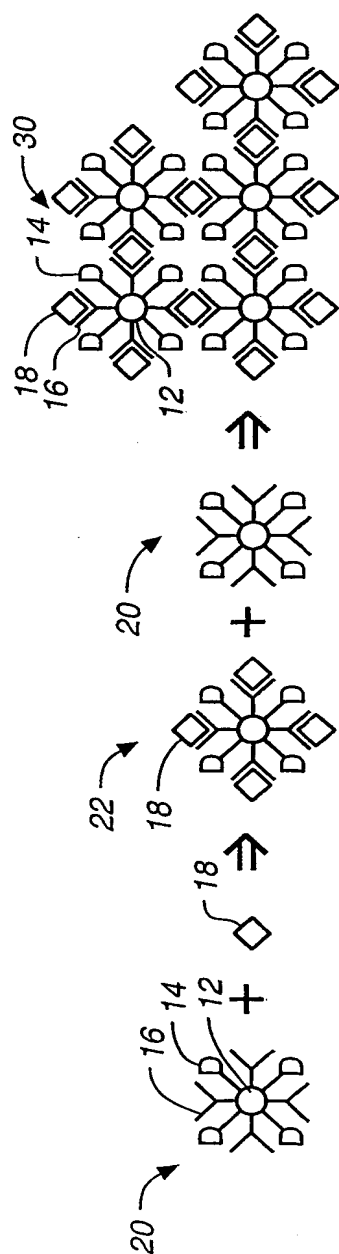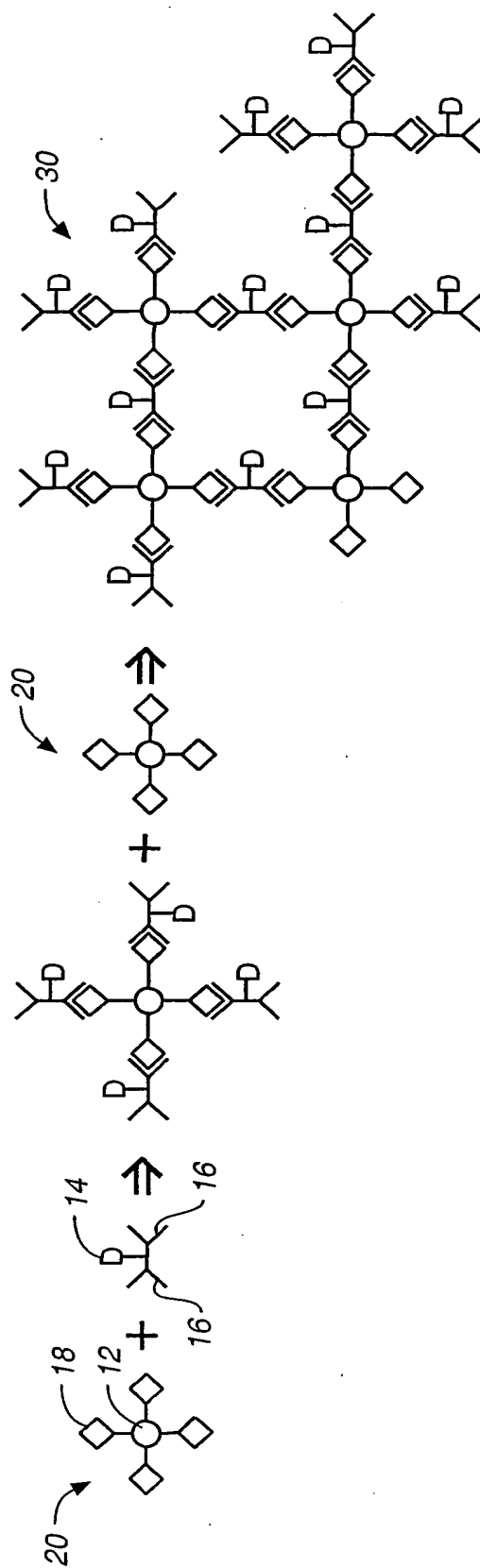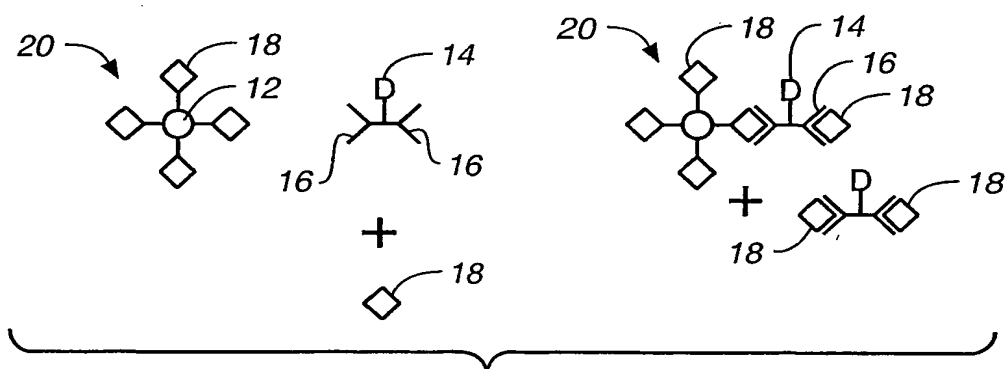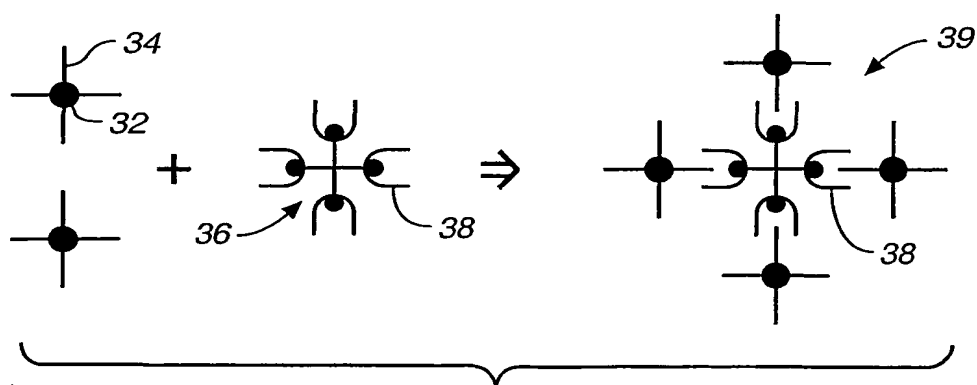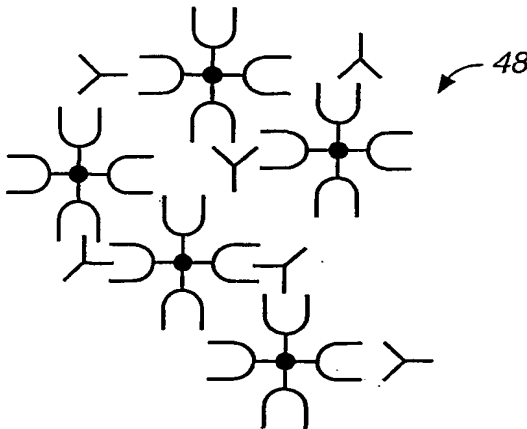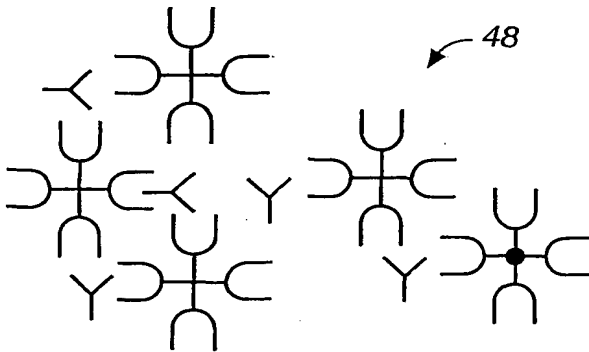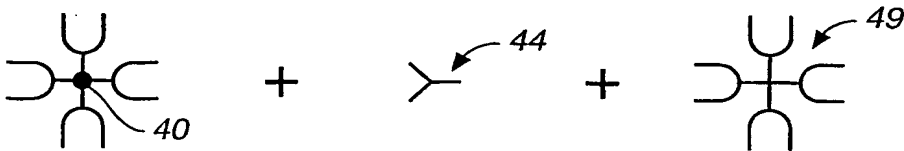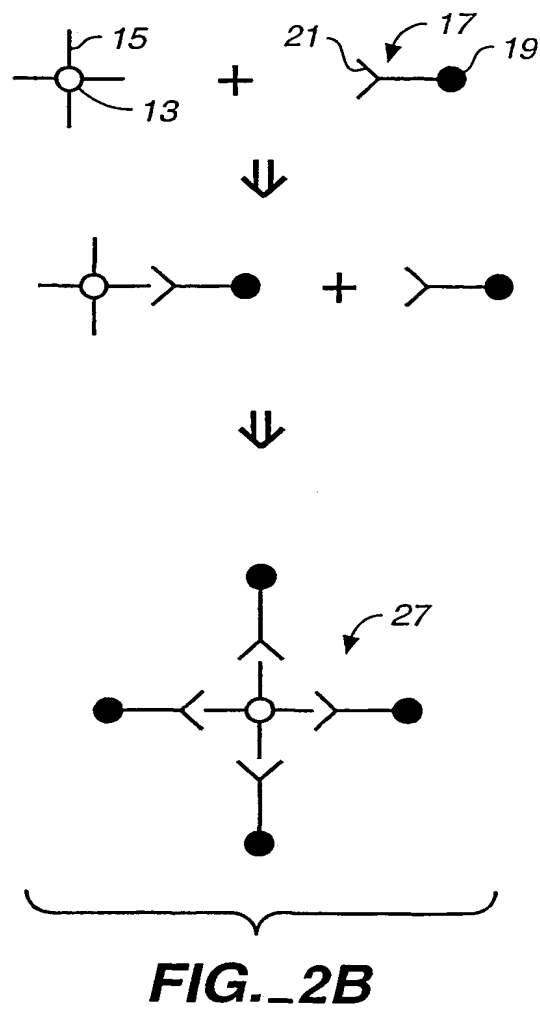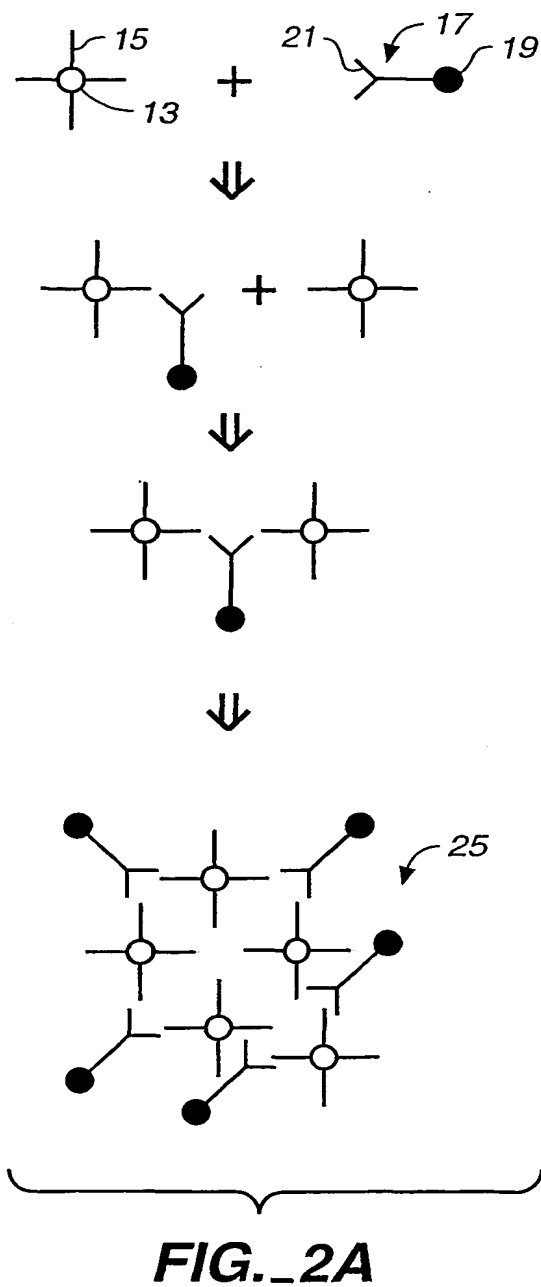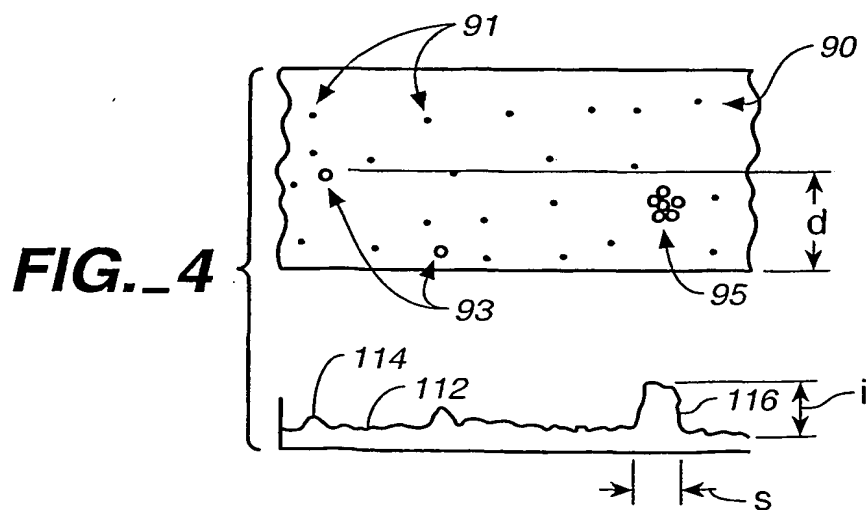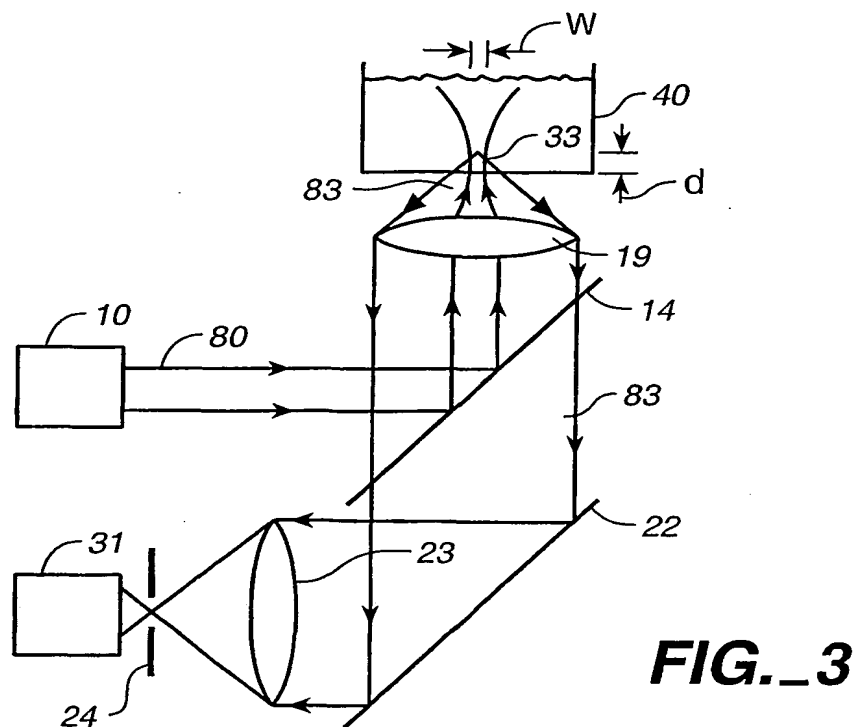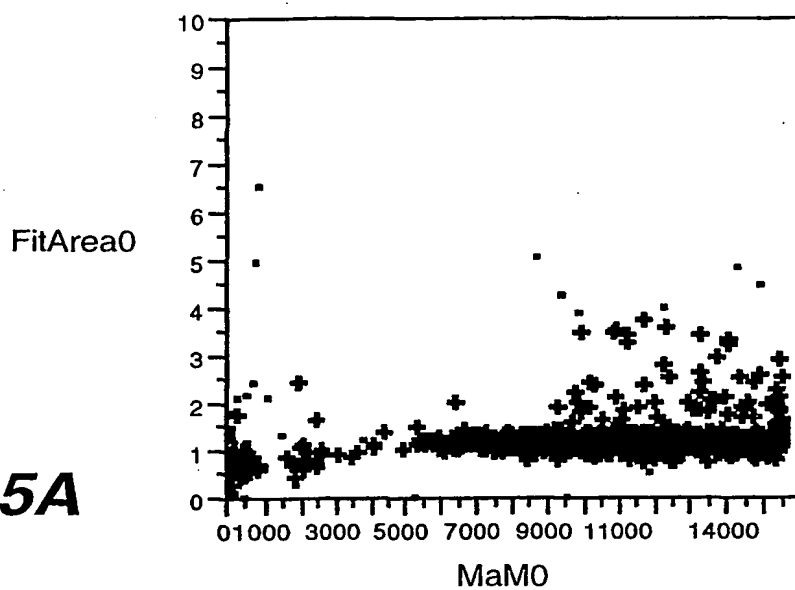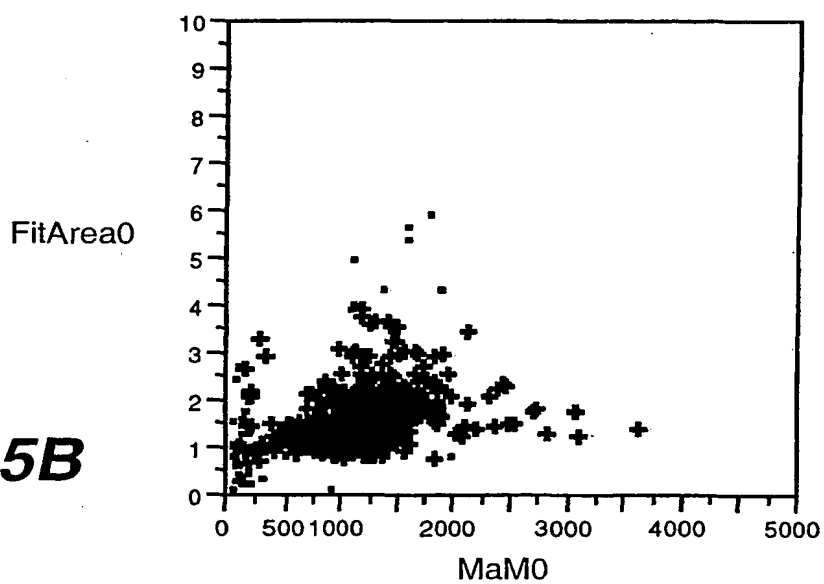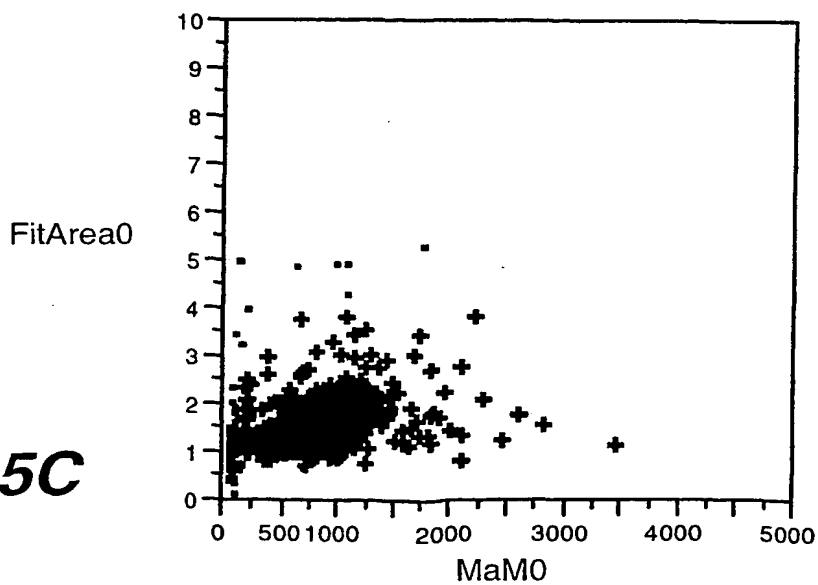
FIG._1A

FIG._1B

FIG.—1C

FIG.—1D

FIG._1E

FIG._1F

FIG._2A

FIG._2B

FIG._3

FIG._4

FIG._5A

FIG._5B

FIG._5C

FIG._5D

FIG._5E

FIG._5F

FIG._6

FIG._7A

FIG._7B

FIG._7C

FIG._7D

FitArea0

FIG._8A

MaM0

FitArea0

FIG._8B

MaM0

FitArea0

FIG._8C

MaM0

FitArea0

FIG._8D

MaM0

FIG._9A

FIG._9B

FIG._9C

FitArea0

FIG._9D

MaM0

FitArea0

FIG._9E

MaM0

FitArea0

FIG._10A

MaM0

FitArea0

FIG._10B

MaM0

FitArea0

FIG._10C

MaM0

FitArea0

FIG._10D

MaM0

FitArea0

FIG._10E

MaM0

FitArea0

FIG._10F

MaM0

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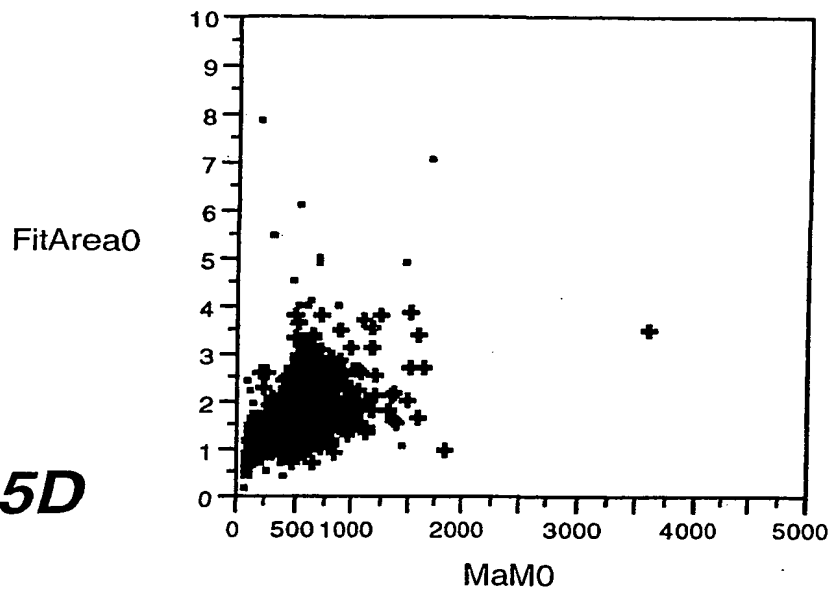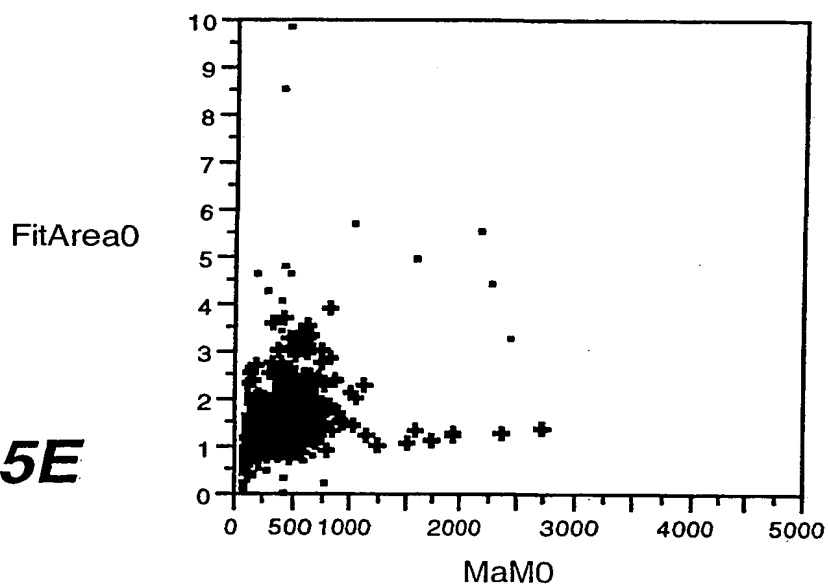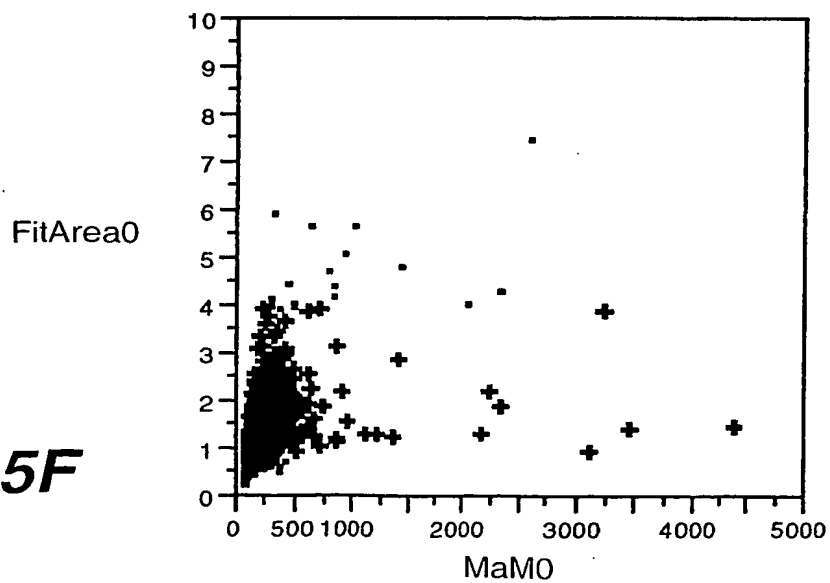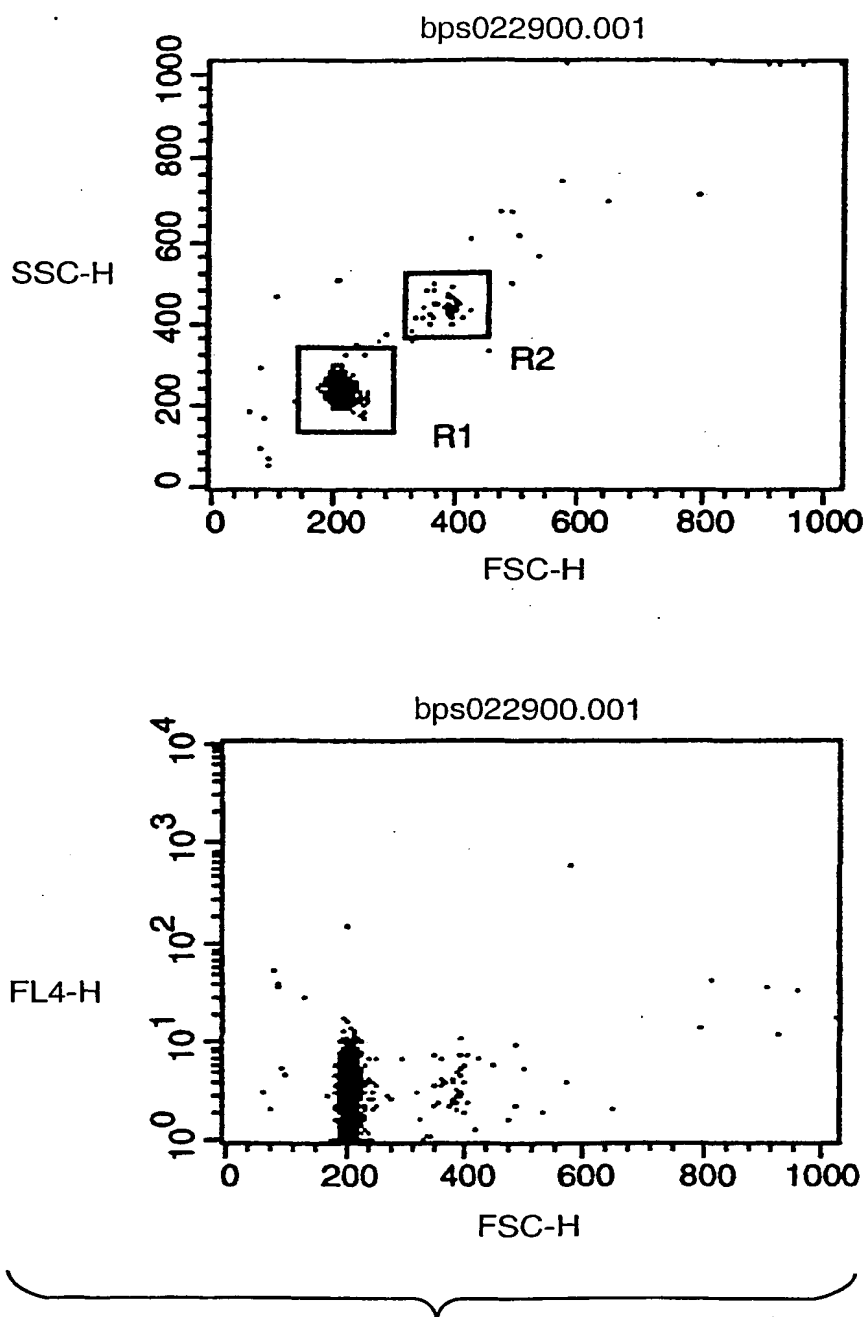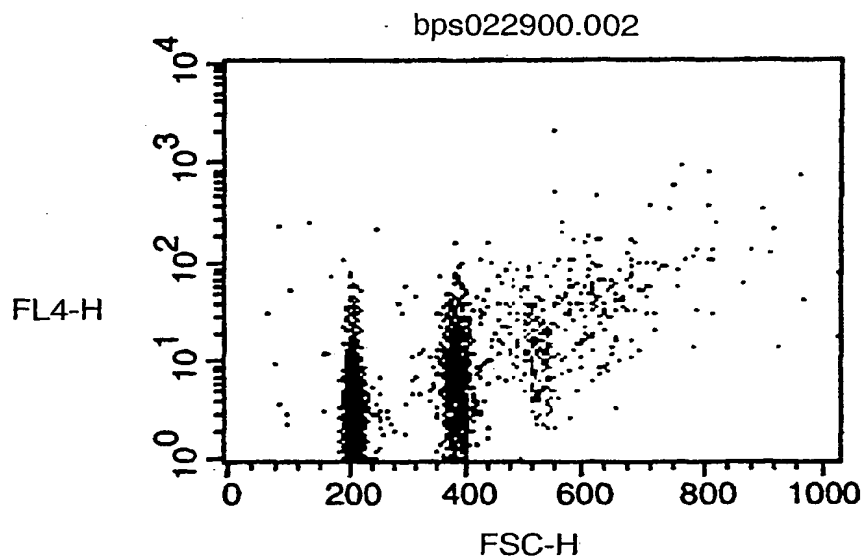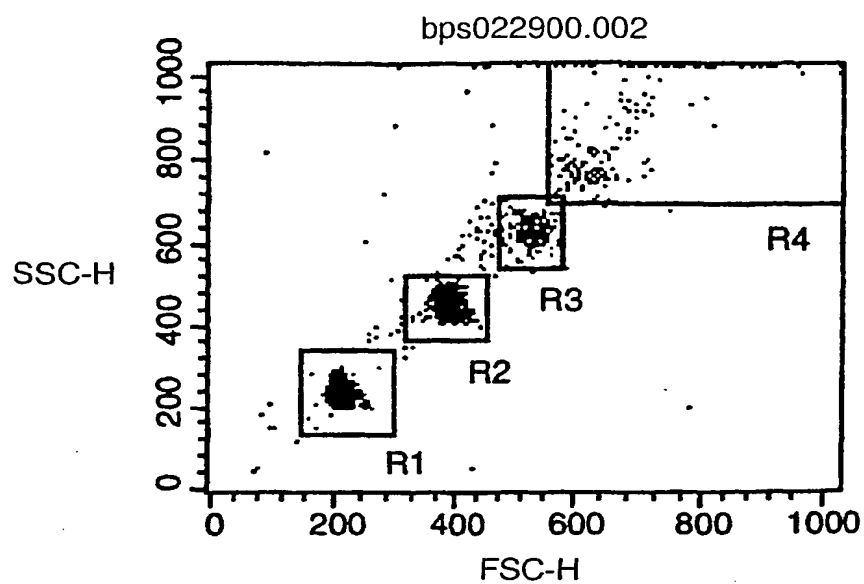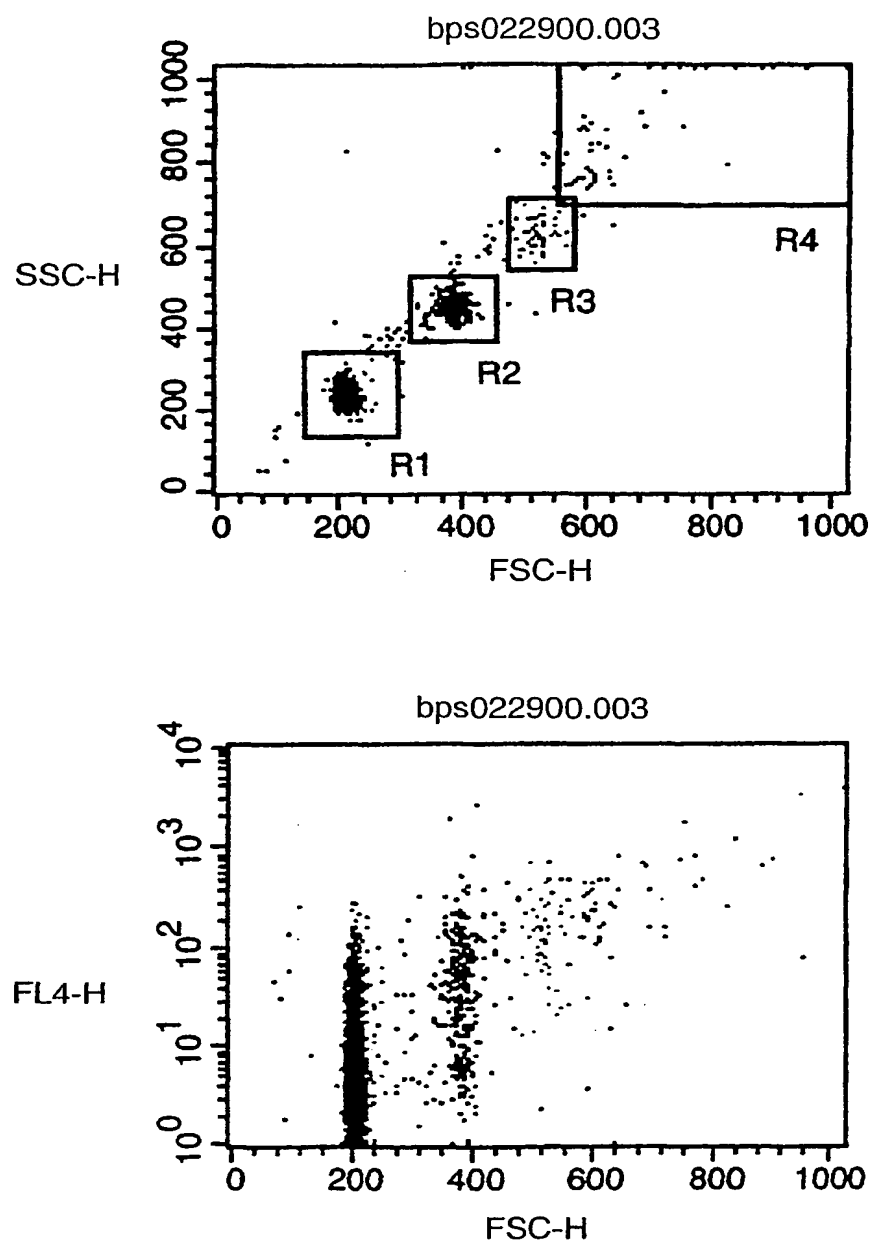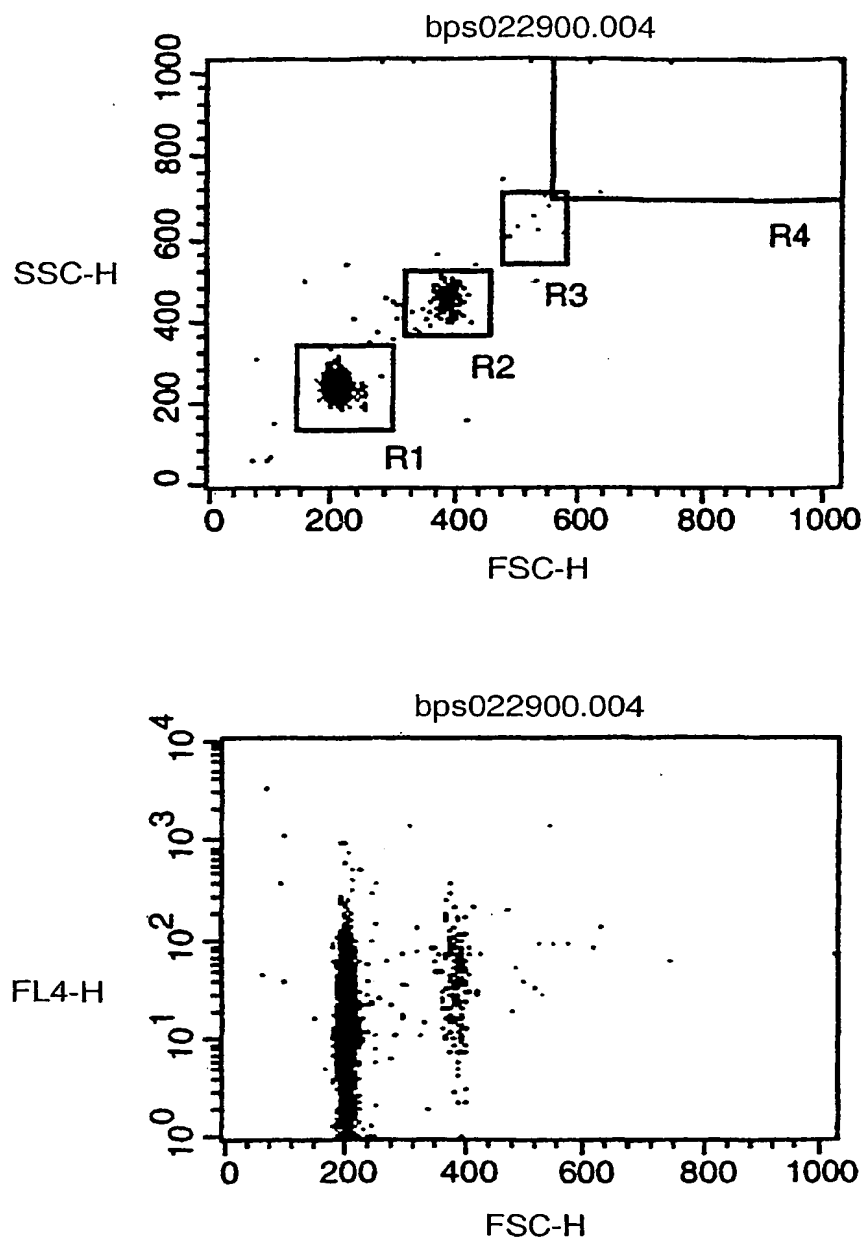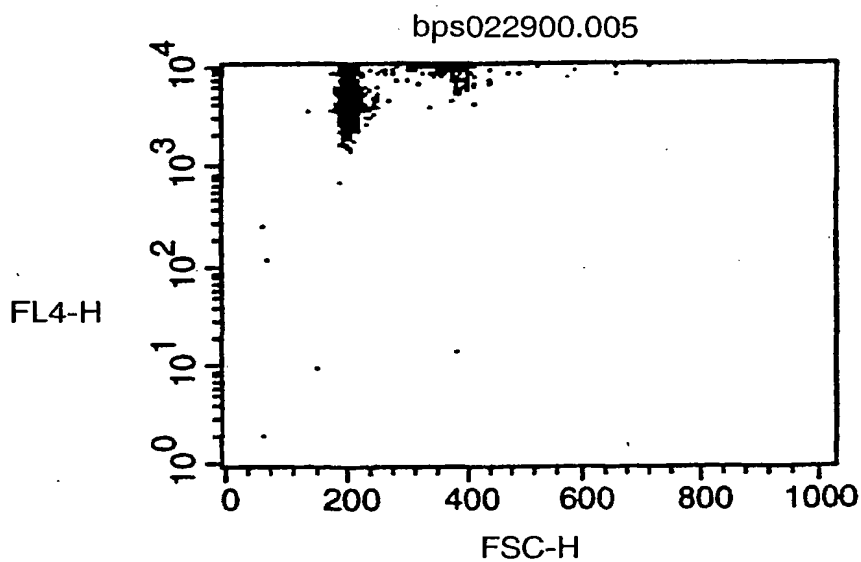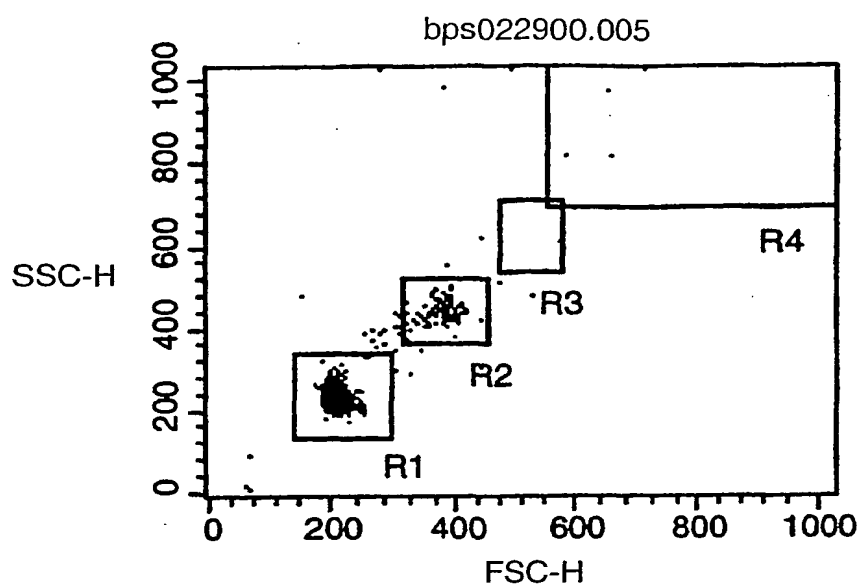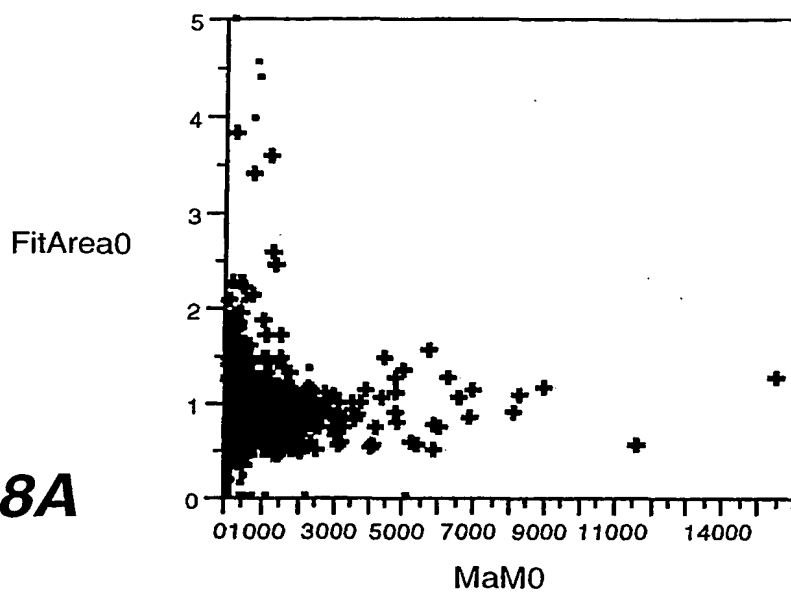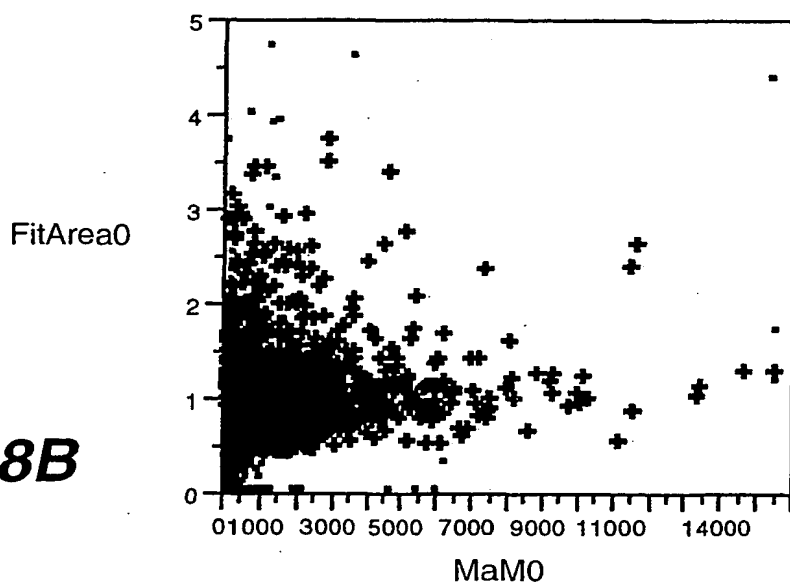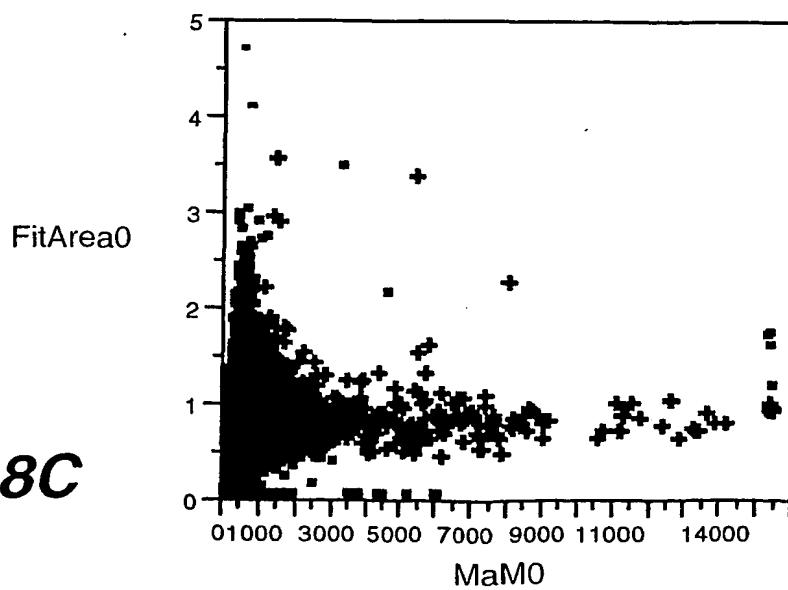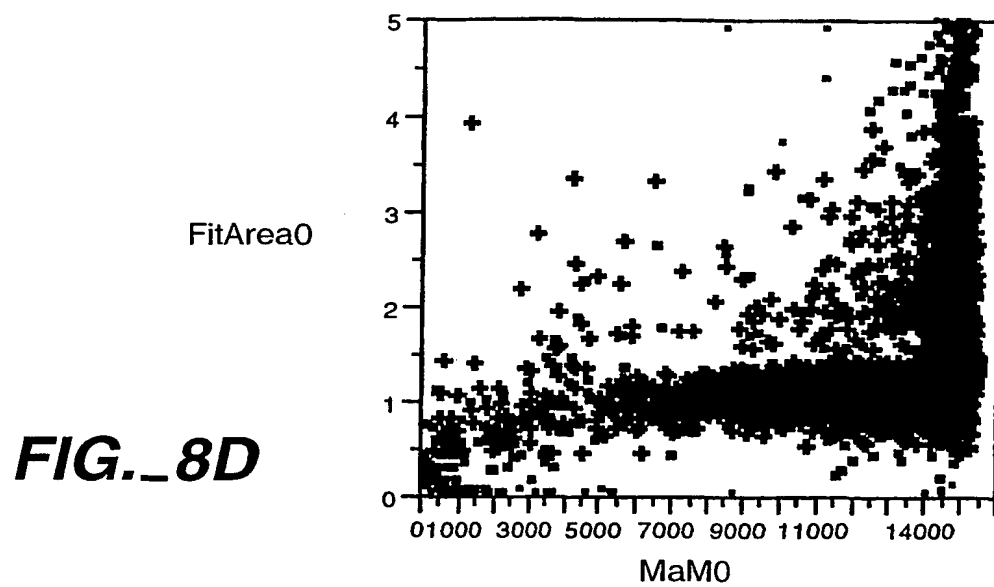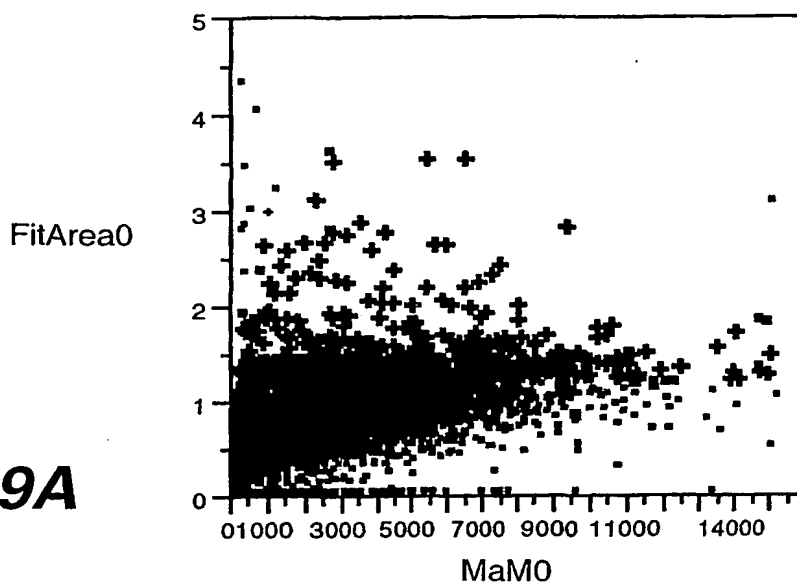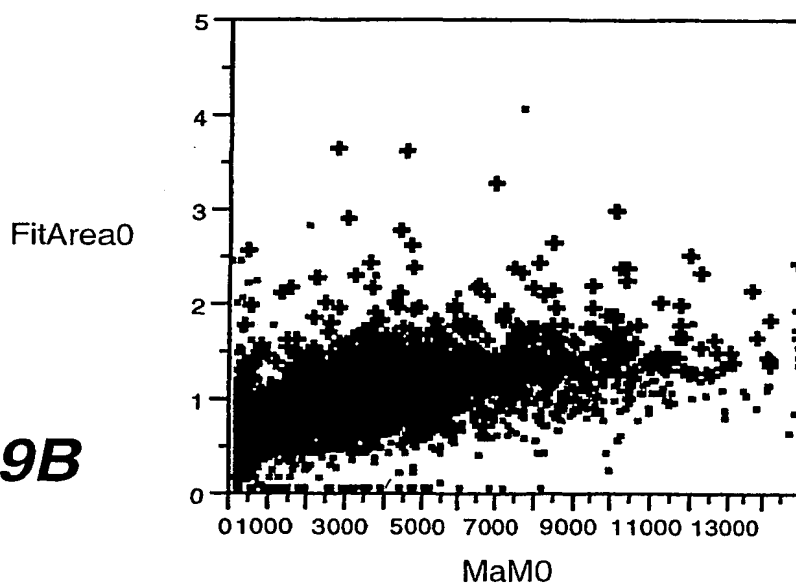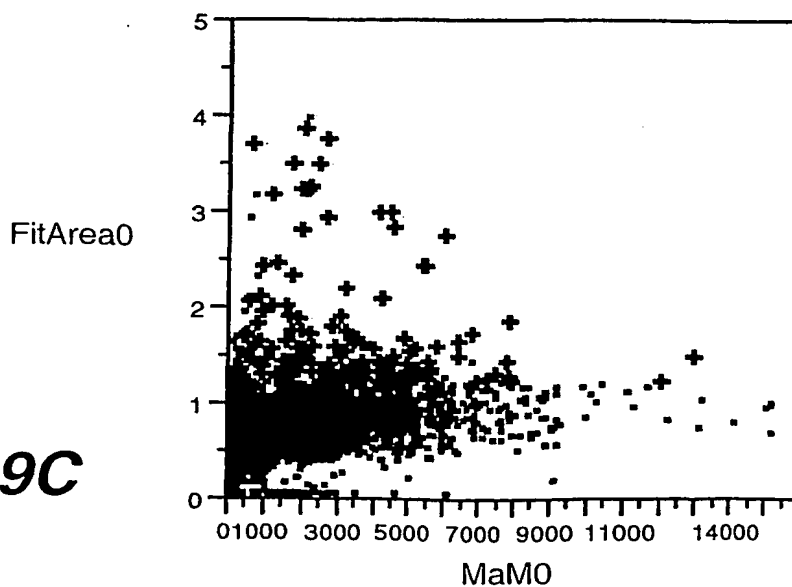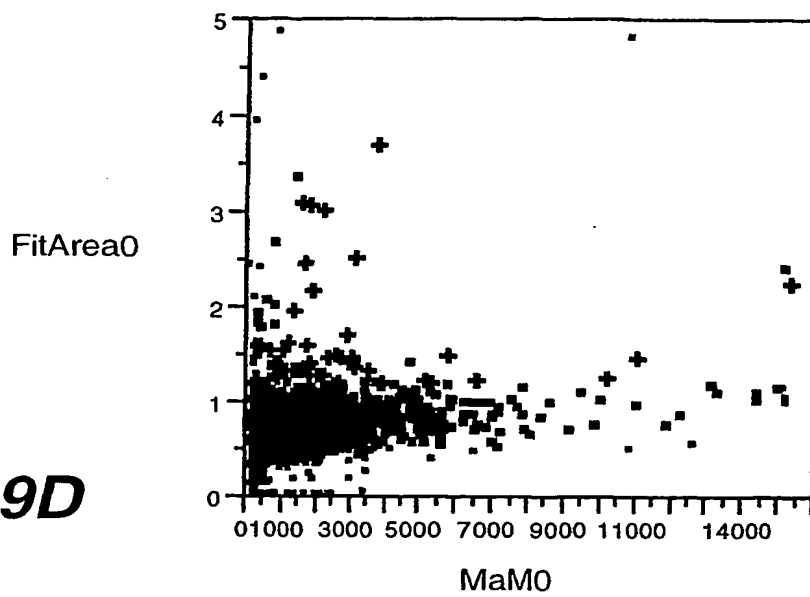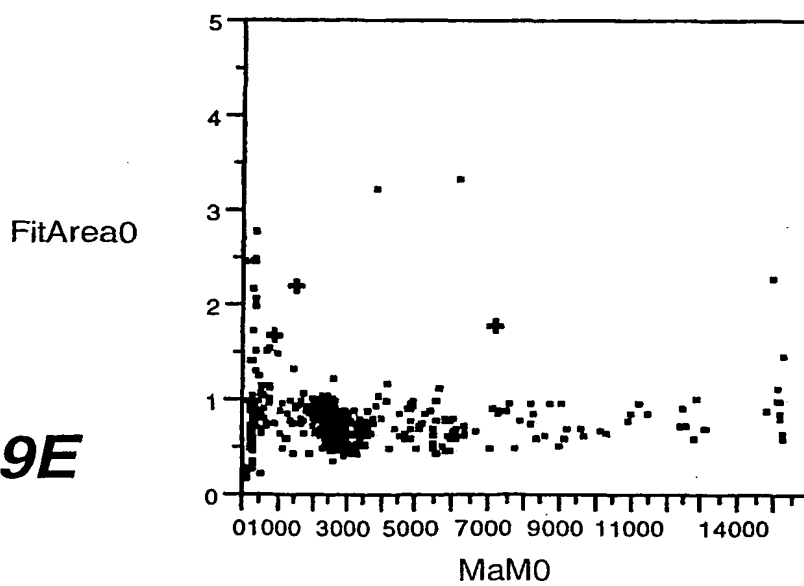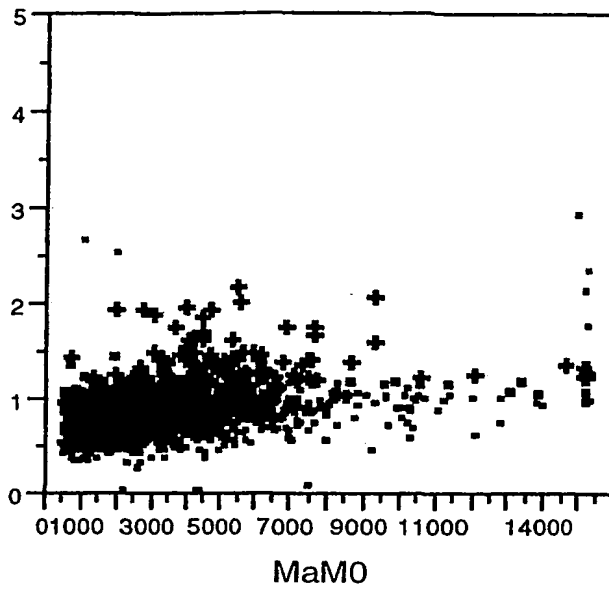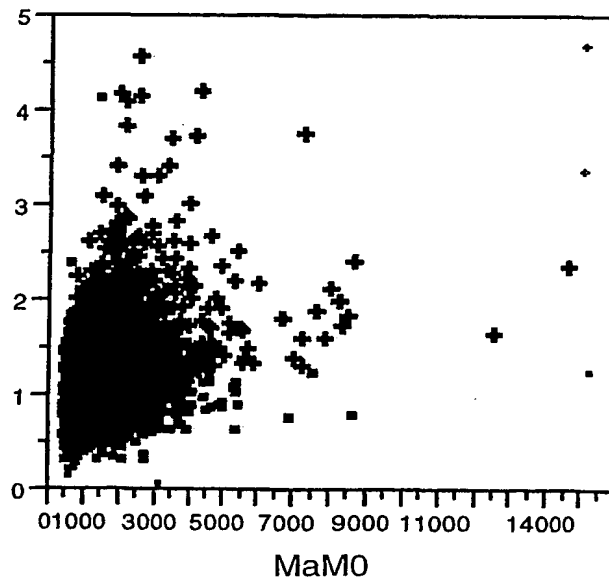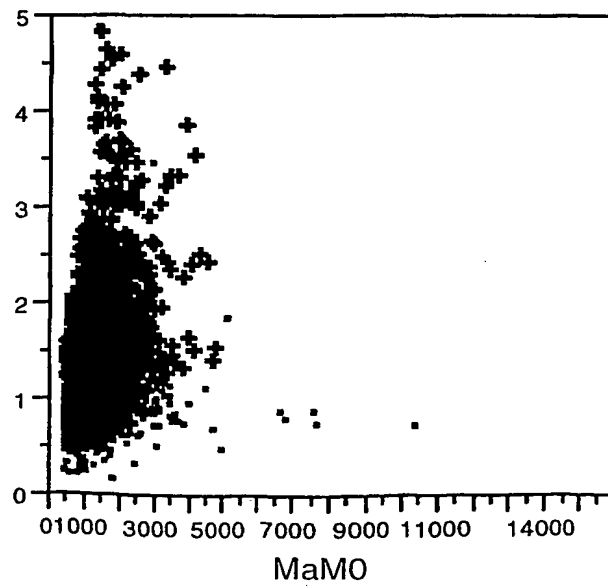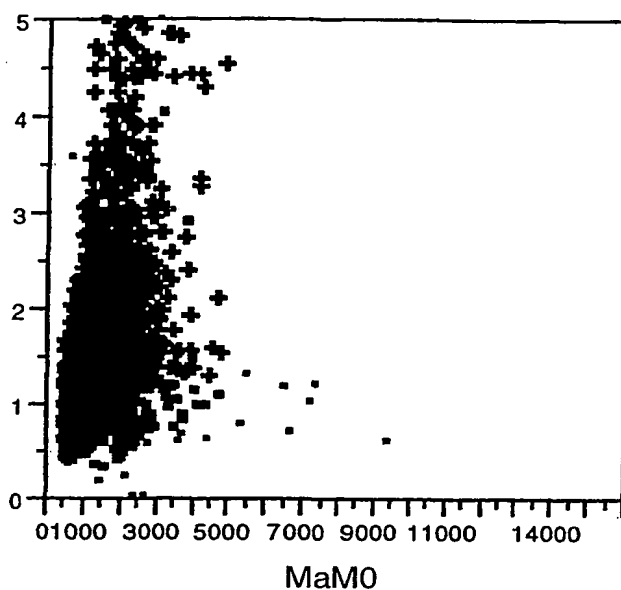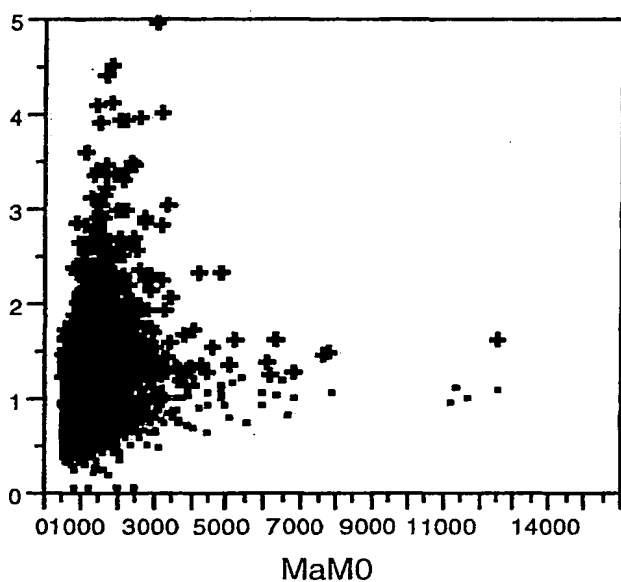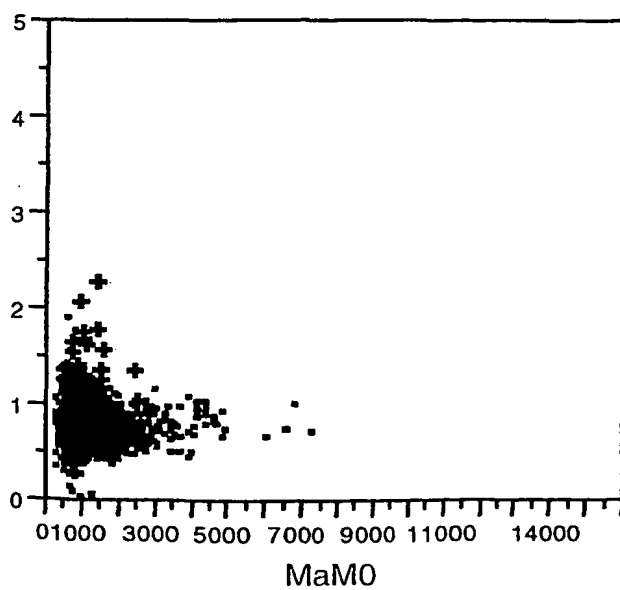
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(81) **Designated States** *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for all designations*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

Published:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** AGGREGATION-BASED ASSAYS

(57) **Abstract:** A method for measuring aggregation by the detection of individual particles (30) is disclosed. The method is adaptable for screening compounds, detection of low levels of a target (e.g. small molecules, large molecules, cells, or microbes), competitive binding assays and other assays. If the assay mixture contains a targeted compound (18), the particulate substrate (20) will aggregate to form a macroparticle (30). One of the components of the assay mixture is labeled with an optically detectable label (14). The macroparticle (30) has an associated macroparticle optical signal which would be identified by optical analysis of the assay mixture.

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :G01N 33/558, 33/547, 33/574
US CL   :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   436/514, 518, 523, 524, 164, 166; 435/7.23, 970, 975; 422/50, 99, 101, 82.05

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, EAST, PALM

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,489,537 A (VAN AKEN) 6 February 1996(06.02.1996), col. 1, line 55-col. 15, line 5.	1-12
A	US 5,491,095 A (BEPKO et al.) 13 February 1996(13.02.1996), col. 1, line 65-col. 2, line 55.	1-12
A	US 5,939,021 A (HANSEN et al.) 17 August 1999(17.08.1999), col. 3, line 54-col. 4, line 44.	1-12
A	US RE36054 (BLAKE et al.) 19 January 1999(19.01.1999), col. 2, line 10-col. 3, line 15.	1-12

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 OCTOBER 2001	31 MAR 2003

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,798,215 A (CATHEY et al.) 25 August 1998(25.08.1998), col. 2, line 64-col. 3, line 23.	1-12

Form PCT/ISA/210 (continuation of second sheet) (July 1998)★

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/514, 518, 523, 524, 164, 166; 435/7.23, 970, 975; 422/50, 99, 101, 82.05